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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : David J. Pinsky et al.

Serial No. : 08/721,447

Filed : September 27, 1996

For : METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

1185 Avenue of the Americas
New York, New York 10036
February 25, 1997

Assistant Commissioner for Patents
Washington, D.C. 20231

Attn: Box Missing Parts

Sir:

COMMUNICATION IN RESPONSE TO DECEMBER 9, 1996 NOTICE TO FILE MISSING PARTS OF APPLICATION UNDER 37 C.F.R. § 1.53(d) AND PETITION FOR ONE MONTH EXTENSION OF TIME

This Communication is submitted in response to the December 9, 1996 Notice To File Missing Parts Of Application under 37 C.F.R. § 1.53(d) issued by the U.S. Patent and Trademark Office in connection with the above-identified application. A copy of the December 9, 1996 Notice is attached hereto as Exhibit A. Applicants hereby submit their executed Declaration and Power of Attorney pursuant to 37 C.F.R. § 1.53(d) and in compliance with 37 C.F.R. § 1.63 as Exhibit B. The Declaration refers to the application's above-identified serial number and filing date.

A response to the December 9, 1996 Notice was originally due February 9, 1997. Applicants hereby request a one month extension of time. Applicants note that, contrary to the December 9, 1996 Notice, small entity status has been established and such status is still applicable. Applicants attach hereto a courtesy copy of the September 27, 1996 Verified Statement (Declaration) Claiming Small Entity Status Under 37 C.F.R. § 1.9(f) And § 1.27(d) - Nonprofit Organization as Exhibit C. The required fee for a one month extension of time for a small entity is FIFTY FIVE DOLLARS ($55.00). The surcharge for submitting the

Declaration and paying any fees under 37 C.F.R. § 1.16(e) is SIXTY FIVE DOLLARS ($65.00) for a small entity. The additional claim fee as required by the December 9, 1996 Notice is ELEVEN DOLLARS ($11.00). A check in the amount of $131.00 is enclosed. Therefore, a response to the December 9, 1996 Notice is now due March 9, 1997. Since March 9, 1997 is a Sunday, under 37 C.F.R. § 1.7 a response to the December 9, 1996 Notice is due the next business day, i.e., March 10, 1997. Accordingly, this Communication is being timely filed.

No fee, other than the $55.00 fee for a one month extension of time, the $65.00 surcharge fee and the $11.00 additional claim fee, is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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I hereby certify that this paper is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

2/25/97
John P. White Date
Reg. No. 28,678

Dkt. 51917/JPW/JML/SKS

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that we, David J. Pinsky, David M. Stern, E. Sander Connolly, Jr., Eric A. Rose, Ann M. Schmidt, Robert A. Solomon and Charles J. Prestigiacomo

have invented certain new and useful improvements in METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

of which the following is a full, clear and exact description.

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Docket 0575/51917/JPW/JML/SKS

METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Treatment of ischemic disorders has been the focus of research for many years. The recent availability of transgenic mice has led to a burgeoning number of reports describing the effects of specific gene products on the pathophysiology of stroke. Although focal cerebral ischemia models in rats have been well-described, descriptions of a murine model of middle cerebral artery occlusion are scant, and sources of potential experimental variability remain undefined.

Acute neutrophil recruitment to postischemic cardiac or pulmonary tissue has deleterious effects in the early reperfusion period, but the mechanisms and effects of neutrophil influx in the pathogenesis of evolving stroke remains controversial.

Summary of the Invention

The present invention provides for a method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of a selectin antagonist in a sufficient amount over a sufficient time period to prevent white blood cell accumulation so as to treat the ischemic disorder in the subject. The invention further provides a method for treating an ischemic disorder in a subject which comprises administering to the subject carbon monoxide gas in a sufficient amount over a sufficient period of time thereby treating the ischemic disorder in the subject. The invention further provides a method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of inactivated Factor IX in a sufficient amount over a sufficient period of time to inhibit coagulation so as to treat the ischemic disorder in the subject.

Brief Description of the Figures

Figure 1. Neutrophil accumulation following focal cerebral ischemia and reperfusion in the mouse. Right middle cerebral artery occlusion was performed for 45 minutes, followed by 23 hours of reperfusion in male C57Bl/J6 mice. One hour prior to middle cerebral artery occlusion, $\approx 3.3 \times 10^5$ ^{111}In-labeled neutrophils were injected into the tail vein. Ipsilateral (right hemispheric) and contralateral (left hemispheric) counts were obtained and normalized per gm of tissue. (n=7, **=p < 0.01).

Figure 2A, 2B, 2C and 2D. Effect of preoperative neutrophil depletion on indices of stroke outcome. C57Bl/J6 male mice were subjected to transient middle cerebral artery occlusion as described above (Wild Type, n=16), and compared with a similar procedure performed in mice immunodepleted of neutrophils during the three days prior to the day of surgery (PMN -, n=18). **Fig. 2A.** Infarct volumes, calculated based on TTC stained serial cerebral sections, and expressed as the % ipsilateral hemispheric volume. **Fig. 2B.** Neurologic deficit score, graded prior to anesthesia 24 hours following transient middle cerebral artery occlusion; 4 represents the most severe neurologic deficit. **Fig. 2C.** Cerebral blood flow, measured by laser doppler flow measurements 2 mm posterior to the bregma, expressed as % contralateral hemispheric blood flow. **Fig 2D.** Mortality at 24 hours following transient middle cerebral artery occlusion. (* = p< 0.05, ** = p< 0.01, *** = p< 0.001).

Figure 3. Expression of Intercellular Adhesion Molecule-1 (ICAM-1) transcripts 24 hours following middle cerebral artery occlusion. RNA was prepared from the ipsilateral (infarct) and the contralateral (noninfarct) hemispheres from the same mouse, and an agarose gel was loaded with 20 μg of total RNA per lane. After overnight transfer to a nylon membrane, the Northern blot was probed with a ^{32}P-labeled 1.90 kb murine ICAM-1 cDNA[33]. A β-actin

probe was used for a control.

Figures 4A and 4B. Expression of Intercellular Adhesion Molecule-1 (ICAM-1) antigen in the cerebral microvasculature 24 hours following middle cerebral artery occlusion. A coronal section of brain was obtained for ICAM-1 immunostaining, so that the noninfarcted and infarcted hemispheres from the same brain could be compared under identical staining conditions. Staining was performed using a rat anti-murine ICAM-1 antibody, with sites of primary antibody binding visualized by alkaline phosphatase. **Fig. 4A.** Cerebral microvessel in the contralateral (noninfarcted) section of a brain obtained 24 hours after middle cerebral artery occlusion. **Fig 4B.** Cerebral microvessel from the ipsilateral (infarcted) hemisphere from the same section of brain as shown in Fig 4A. Endothelial cells from ipsilateral cerebral microvessels demonstrate increased expression of ICAM-1 (bright red staining). Magnification 250X.

Figures 5A and 5B. Cerebrovascular anatomy in homozygous null ICAM-1 mice (Fig. 5B) and wild type controls (Fig. 5A). India ink staining of cerebrovascular anatomy with an inferior view of the Circle of Willis demonstrates that there were no gross anatomic differences in the vascular pattern of the cerebral circulation, with intact posterior communicating arteries in both.

Figures 6A and 6B. TTC-stained serial sections at 24 hours from representative wild type (Fig. 6A) or homozygous null ICAM-1 mice (Fig. 6B) subjected to transient middle cerebral artery occlusion. The pale white area in the middle cerebral artery territory represents infarcted brain tissue, whereas viable tissue stains brick red. Quantification of infarct volumes by planimetry of serial cerebral sections in multiple experiments is shown in Figure 7A.

Figures 7A, 7B, 7C and 7D. Role of ICAM-1 in stroke outcome.

Transient middle cerebral artery occlusion was performed as described in ICAM-1 +/+ (Wild Type, n=16) or ICAM-1-/- (n=13) mice, and indices of stroke outcome measured as described in Figure 2. **Fig. 7A.** Effect of ICAM-1 on infarct volume, **Fig. 7B.** neurologic deficit score, **Fig. 7C.** cerebral blood flow, and **Fig. 7D.** mortality. (* = p< 0.05, ** = p< 0.01).

Figures 8A and 8B. Effect of hypoxia on Weibel-Palade body exocytosis. **Fig. 8A.** Human umbilical veins were exposed to hypoxia (pO_2 15-20 Torr) or normoxia for the indicated durations, and vonWillebrand factor (vWF) secretion quantified by ELISA. ***=p<0.001 for hypoxia vs normoxia. **Fig 8B.** Similar experiments were performed for 8 hrs in the presence of 2 mM Ca^{++} (Ca^{++} 2 mM), 0 mM Ca^{++} (Ca^{++}-free), or 0 mM Ca^{++} with 2 mM EGTA added to chelate residual extracellular Ca^{++} (Ca^{++}-free + EGTA).

Figures 9A, 9B and 9C. Effect of endothelial hypoxia on P-selectin expression and neutrophil adhesion. **Fig 9A.** P-selectin expression on HUVECs exposed to normoxia or hypoxia, determined by specific binding of radiolabelled monoclonal anti-P-selectin IgG (WAPS12.2 clone). Data are expressed as relative binding compared with the 4 hr normoxic time point. **Fig 9B.** Effect of inhibiting protein synthesis on hypoxia-induced P-selectin expression. In a separate experiment, the effect of cyloheximide (10 μg/mL, + CHX) added at the start of the 4 hour normoxic or hypoxic period on P-selectin expression is shown. Comparison is made to simultaneous experiments performed in the absence of cyloheximide (-CHX), with data expressed as relative binding compared with normoxic (-CHX) binding. Means ± SEM are shown; *=p<0.05 vs normoxia (-CHX); †=p<0.05 vs normoxia (+CHX). **Inset:** Effect of cyloheximide (10 μg/mL) on protein synthesis at 4 hrs, measured as trichloroacetic acid-precipitable ^{35}S-labeled proteins following ^{35}S-methionine and ^{35}S-cysteine administration. **Fig 9C.** ^{111} Indium-labeled neutrophil binding to normoxic (N) or hypoxic (H) human umbilical vein endothelial monolayers at 4 hrs, in the presence of a blocking

anti-P-selectin antibody (WAPS 12.2 clone) or a nonblocking anti-P-selectin antibody (AC1.2 clone). Means ± SEM are shown; **=p<0.01.

Figures 10A, 10B and 10C. Role of neutrophils and endothelial P-selectin in rodent cardiac preservation followed by heterotopic transplantation. **Fig 10A.** Rat cardiac preservation. Hearts were transplanted immediately after harvest (Fresh, n=8) or preserved for 16 hrs in lactated Ringer's solution at 4 °C followed by transplantation (Prsvd, n=4). The effect of administering non-blocking anti-P-selectin antibody (AC1.2, n=3), immunodepleting recipients of neutrophils prior to donor heart implantation (- PMN, n=4), or administering 250 μg of blocking anti-P-selectin IgG (n=4) 10 minutes prior to reperfusion on cardiac graft survival (shaded bars) and leukostasis (myeloperoxidase activity, dark bars). Means ± SEM are shown; For graft survival, c vs a, p<0.0001; g vs c, p<0.05; i vs e or c, p<0.05. For graft neutrophil infiltration, d vs b, p<0.01; h vs d, p<0.05; j vs d or f, p<0.05. **Fig 10B.** Role of coronary endothelial P-selectin in cardiac preservation, using donor hearts from P-selectin null (or wild type control) mice that were flushed free of blood prior to preservation. Graft survival was assessed by the presence/absence of cardiac electrical/mechanical activity exactly ten minutes following reestablishment of blood flow. **Fig 10C:** Quantification of neutrophil infiltration by measurement of myeloperoxidase activity (dABs 460 nm/min) as described[15,18]. (For bars shown from left to right, n=14, 8,13, and 7, respectively, with P values indicated).

Figures 11A and 11B. Weibel-Palade body release during human cardiac surgery in 32 patients. **Fig 11A.** Coronary sinus blood was sampled at the start (CS_1) and conclusion (CS_2) of the ischemic period (aortic cross-clamping). ELISAs were performed for thrombomodulin (TM) and vWF. **Fig 11B.** vWF immunoelectrophoresis of a representative sample of CS_1 and CS_2 blood from the same patient (dilution factors are indicated). There is an increase in

high molecular weight multimers detected in the CS_2 samples.

Figures 12A, 12B, 12C and 12D. Overview of operative setup for murine focal cerebral ischemia model. **Fig 12A.** Suture based retraction system is shown in the diagram. **Fig 12B.** View through the operating microscope. The large vascular stump represents the external carotid artery, which is situated inferomedially in the operating field. **Fig 12C.** Photograph of heat-blunted occluding suture of the indicated gauge (5-0 [bottom] or 6-0 nylon [top]). **Fig 12D.** Schematic diagram of murine cerebrovascular anatomy, with thread in the anterior cerebral artery, occluding the middle cerebral artery at its point of origin.

Figure 13. Comparison of cerebrovascular anatomy between strains of mice. Following anesthesia, mice were given an intracardiac injection of India ink followed by humane euthanasia. An intact Circle of Willis can be observed in all strains, including bilateral posterior communicating arteries, indicating that there are no gross strain-related differences in cerebrovascular anatomy.

Figures 14A, 14B and 14C. Effects of mouse strain on stroke outcome. Mice (20-23 gm males) were subjected to 45 minutes of MCA occlusion (using 12mm 6.0 occluding suture) followed by 24 hours of reperfusion, and indices of stroke outcome determined. **Fig 14A.** Effects of strain on infarct volume, determined as a percentage of ipsilateral hemispheric volume, as described in the Methods section. **Fig 14B.** Effects of strain on neurological deficit score, graded from no neurologic deficit (0) to severe neurologic deficit (4), with scores determined as described in the Methods section. **Fig 14C.** Effects of strain on cerebral blood flow, measured by laser doppler flowmetry as relative flow over the infarcted territory compared with blood flow over the contralateral (noninfarcted) cortex. Strains included 129J (n=9), CD1 (n=11), and C57/Bl6 mice (n=11); *= $p < 0.05$ vs 129J mice.

Figures 15A, 15B and 15C. Effects of animal size and diameter of the occluding suture on stroke outcome. Male CD-1 mice of the indicated sizes were subjected to middle cerebral artery occlusion (45 minutes) followed by reperfusion (24 hours) as described in the Methods section. Suture size (gauge) is indicated in each panel. Small animals (n=11) were those between 20-25 gm (mean 23 gm), and large animals were between 28-35 gm (mean 32 gm; n=14 for 6.0 suture, n=9 for 5.0 suture). **Fig 15A.** Effects of animal/suture size on infarct volume, **Fig 15B.** neurological deficit score, and **Fig 15C.** cerebral blood flow, measured as described in Figure 14. P values are as shown.

Figures 16A, 16B and 16C. Effects of temperature on stroke outcome. Male C57/Bl6 mice were subjected to 45 minutes of MCA occlusion (6.0 suture) followed by reperfusion. Core temperatures were maintained for 90 minutes at 37°C (normothermia, n=11) using an intrarectal probe with a thermocouple-controlled heating device. In the second group (hypothermia, n=12), animals were placed in cages left at room temperature after an initial 10 minutes of normothermia (mean core temperature 31°C at 90 minutes). In both groups, after this 90 minute observation period, animals were returned to their cages with ambient temperature maintained at 37°C for the duration of observation. Twenty-four hours following MCA occlusion, indices of stroke outcome were recorded; **Fig 16A.** infarct volume, **Fig 16B.** neurological deficit score, and **Fig 16C.** cerebral blood flow, measured as described in Figure 3. * = $p<0.05$ values are as shown.

Figures 17A, 17B and 17C. Outcome comparisons between permanent focal cerebral ischemia and transient focal cerebral ischemia followed by reperfusion. The MCA was either occluded permanently (n=11) or transiently (45 minutes, n=17) with 6.0 gauge suture in 22 gram Male C57/Bl6 mice, as described in the Methods section. Twenty-four hours following MCA occlusion, indices of stroke outcome were recorded; **Fig 17A.** infarct volume, **Fig 17B.**

neurological deficit score, and **Fig 17C.** cerebral blood flow, measured as described in Figure 14.

Figures 18A and 18B. P-selectin expression and neutrophil (PMN) accumulation following middle cerebral artery occlusion (MCAO) in mice. **Fig 18A.** P-selectin expression following MCAO and reperfusion. Relative expression of P-selectin antigen in the ipsilateral cerebral hemisphere following middle cerebral artery occlusion was demonstrated using either a ^{125}I-labeled rat monoclonal anti-P-selectin IgG or a ^{125}I-labeled nonimmune rat IgG to control for nonspecific extravasation. Experiments were performed as described in the legend to figure 18. Values are expressed as ipsilateral cpm/contralateral cpm. n = 6 for each group, except for control 30 min (n = 4); ‡ = $p < 0.001$, 30 min reperfusion vs immediate pre-occlusion; * = $p < 0.025$, change in P-selectin accumulation vs change in control IgG accumulation. **Fig 18B.** Time course of PMN accumulation following focal cerebral ischemia and reperfusion in the mouse. For these experiments, ≈3.3 x 10^5 ^{111}In-labeled PANS were injected intravenously into PS wild type (PS +/+) mice 15 minutes prior to middle cerebral artery occlusion (MCAO). ^{111}In-PMN accumulation was measured immediately following sacrifice as the ratio of ipsilateral/contralateral cpm under the following experimental conditions: prior to MCAO (Pre-O, n = 4), immediately following MCAO (Post-O, n = 6), and 10 minutes following MCAO but still prior to reperfusion (:10 Post-O, n = 6). To establish the effect of reperfusion on PMN accumulation, reperfusion was initiated following 45 minutes of ischemia. PMN accumulation was measured following 30 minutes (n = 6), 300 minutes (n = 3), and 22 hours (n = 8) of reperfusion. Under identical conditions, PMN accumulation was measured in P-selectin null (PS -/-) mice after 45 minutes of ischemia and 22 hours of reperfusion (n = 7, * = $p < 0.05$ vs 45 min MCAO/22 hrs reperfusion in PS +/+ animals).

Figure 19. Role of P-selectin in the cerebrovascular no-reflow.

Cerebral blood flow was measured in PS +/+ (top panel) and PS -/- (middle panel) mice using a laser doppler flow probe, and expressed as the percentage of contralateral (nonischemic) hemispheric blood flow (± SEM). Blood flow was measured at the following time points: **a**, prior to MCAO (PS +/+, n = 16; PS -/-, n = 23); **b**, immediately following MCAO (PS +/+, n = 42; PS -/-, n = 40); **c**, 10 minutes following MCAO but still prior to reperfusion (PS +/+, n = 36; PS -/-, n = 34); **d**, immediately following reperfusion (PS +/+, n = 36; PS -/-, n = 34); **e**, 30 minutes following reperfusion (PS +/+, n = 8; PS -/-, n = 5); and **f**, 22 hours following reperfusion (PS +/+, n = 15; PS -/-, n = 5). The bottom panel represents an overlay of the top two panels, with error bars omitted for clarity.

Figure 20. Cerebrovascular anatomy in homozygous null P-selectin mice, PS -/- (right) and wild type controls, PS +/+ (left). India ink/carbon black staining of cerebrovascular anatomy with an inferior view of the Circle of Willis demonstrates that there were no gross anatomic differences in the vascular pattern of the cerebral circulation, with intact posterior communicating arteries in both.

Figures 21A, 21B and 21C. Effect of the P-selectin gene on stroke outcomes. Middle cerebral artery occlusion was performed for 45 minutes, followed by 22 hours of reperfusion in P-selectin +/+ (n = 10) or P-selectin -/- (n = 7) mice. Effect of P-selectin on: **Fig 21A.** infarct volume, as evidenced by 2% 2,3,5, triphenyl, 2H-tetrazolium chloride (TTC) staining, and calculated as percent of ipsilateral hemisphere; **Fig 21B.** neurologic deficit score, (1 = normal spontaneous movements; 2 = clockwise circling; 3 = clockwise spinning; 4 = unresponsiveness to noxious stimuli); **Fig 21C.** percent survival at time of sacrifice. (* = $p < 0.05$).

Figures 22A, 22B, 22C and 22D. Effect of P-selectin blockade on stroke outcomes. PS +/+ mice were given either a blocking rat anti-mouse anti-P-selectin IgG (clone RB 40.34, 30 μg/mouse) or a

similar dose of nonimmune rat IgG immediately prior to surgery. **Fig 22A.** Cerebral blood flow at thirty minutes following reperfusion; After 22 hours of reperfusion, infarct volumes **Fig 22B.**, neurological deficit scores **Fig 22C.**, and mortality **Fig 22D.** are shown. (n=7 for each group, *=p<0.05).

Figures 23A and 23B. **Fig 23A.** The effect of carbon monoxide inhalation on cerebral infarct volumes. Mice were placed in bell jars, in which they were exposed to 0.1% CO for 12 hours. After this treatment, they were removed from the bell jars and subjected to intraluminal occlusion of the middle cerebral artery. At 24 hours, animals were sacrificed and infarct volumes measured by triphenyltetrazolium chloride (TTC) staining as shown in Figure 25. Quantification of infarction volumes (mean ± SEM) is expressed as the percent of infarction of the ipsilateral hemisphere. These data show that inhaled CO reduces infarct volumes following stroke. **Fig 23B.** The effect of carbon monoxide inhalation on mortality following stroke. Experiments were performed as described above. Mortality at 24 hours is shown. These data show that inhaled CO reduces mortality following stroke.

Figures 24A and 24B. **Fig 24A.** Dose-response of inhaled carbon monoxide on stroke outcome. Experiments are described above. CO was inhaled at the indicated doses. These data show that inhaled CO reduces infarct volume in a dose-dependent fashion, with 0.1% providing optimal protection. **Fig 24B.** Role of heme oxygenase, the enzyme which makes CO, in stroke. Animals were given either vehicle (DMSO) alone as a control or zinc protoporphyrin IX (ZnPP) or tin protoporphyrin IX (SnPP). In a final group, mice were given biliverdin (Bili), a compound which is formed along with CO during the process of heme degradation by heme oxygenase. Left panel shows infarction volumes. Right panel shows mortality. These experiments demonstrate that when heme oxygenase activity is blocked, stroke outcomes are worse (larger infarcts and higher mortalities). Because biliverdin administration is not protective,

these data suggest that the other coproduct of heme oxygenase activity (CO) is protective.

Figure 25. TTC staining of serial cerebral sections for the animals of Figure 23. Infarcted tissue appears white, and viable tissue appears brick red.

Figures 26A-26F. Effect of focal cerebral ischemia on heme oxygenase I (HO-I) induction. **Figs 26A-26C** show in situ hybridization of HO-I mRNA in stroke **(Fig 26B)** and in controls **(Figs 26A and 26C). Figs 26D-26F** show immunohistochemistry of HO-I protein. **Fig 26E** shows that the protein is expressed in blood vessels and astrocytes following stroke. **Figs 26D and 26F** show that the protein is not expressed in blood vessels and astrocytes in controls.

Figure 27. Effect of focal cerebral ischemia on heme oxygenase I (HO-I) mRNA induction. Contralateral indicates the nonstroke side of the brain. Ipsilateral indicates the brain side subjected to stroke. In both animals, the side of the brain subjected to stroke demonstrates increased HO-I but the nonstroke side does not.

Figure 28 Effect of hypoxia on heme oxygenase I (HO) induction. Mice exposed to a hypoxic environment for 12 hours (to simulate ischemia) show an increase in heme oxygenase I mRNA compared with normoxic controls. These data show a potential mechanism whereby hypoxic pre-exposure can also confer protection against subsequent ischemic events, which was found to be true in mice subjected to hypoxia followed by stroke.

Figure 29 Effect of hypoxia on heme oxygenase I (HO-I) protein expression in mouse brain endothelial cells. Hypoxia causes HO-I protein levels to increase in these brain-derived endothelial cells.

Figure 30 · Effect of hypoxia on heme oxygenase I (HO-I) mRNA induction in mouse brain endothelial cells. Hypoxia causes HO-I mRNA levels to increase in these brain-derived endothelial cells.

Detailed Description of the Invention

The present invention provides for a method for treating an ischemic disorder in a subject which includes administering to the subject a pharmaceutically acceptable form of a selectin antagonist in a sufficient amount over a sufficient time period to prevent white blood cell accumulation so as to treat the ischemic disorder in the subject. The selectin antagonist may be a peptide mimetic, a nucleic acid molecule, a ribozyme, a polypeptide, a small molecule, a carbohydrate molecule, a monosaccharide, an oligosaccharide or an antibody. The selectin may be a P-selectin, an E-selectin, or an L-selectin. The antibody may be a P-selectin antibody. The antibody may further include a polyclonal antibody or a monoclonal antibody. The P-selectin antagonist may include a nitric oxide (NO) precursor such as L-arginine, an NO donor such as nitroglycerin or nitroprusside, a cyclic nucleotide analog such as a cyclic GMP or cyclic AMP analog, or a phosphodiesterase inhibitor.

The pharmaceutically acceptable form of P-selectin antagonist may include a P-selectin antagonist and a pharmaceutically acceptable carrier. The carrier may include an aerosol, intravenous, oral or topical carrier.

The white blood cell may be a neutrophil or a monocyte. The subject may be a mammal. The mammal may be a human, a cow, a pig, a sheep, a dog, a cat, a monkey, a fowl or any animal model of a human disease or disorder.

The ischemic disorder may include, but is not limited to a peripheral vascular disorder, a venous thrombosis, a pulmonary embolus, a myocardial infarction, a transient ischemic attack, unstable angina, a reversible ischemic neurological deficit, sickle cell anemia or a stroke disorder.

The subject may be undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery. The organ transplantation surgery may include heart, lung, pancreas or liver transplantation surgery.

The present invention further provides for a method for treating an ischemic disorder in a subject which comprises administering to the subject carbon monoxide gas in a sufficient amount over a sufficient period of time thereby treating the ischemic disorder in the subject.

The administration of carbon monoxide may be via inhalation by the subject or via extracorporeal exposure to blood or body fluids of the subject.

The amount of carbon monoxide which may be sufficient to treat the subject includes but is not limited to from about 0.0001% carbon monoxide in an inert gas to about 2% carbon monoxide in an inert gas. The inert gas may be oxygen, nitrogen, argon or air. In one embodiment of the present invention, the amount of carbon monoxide administered may be 0.1% carbon monoxide in air.

The period of time sufficient to administer carbon monoxide to a subject to treat an ischemic disorder includes but is not limited to from about 1 day before surgery to about 1 day after surgery. The period of time may be from about 12 hours before surgery to about 12 hours after surgery. The period of time may further include from about 12 hours before surgery to about 1 hour after surgery. The period of time may further include from about 1 hour before surgery to about 1 hour after surgery. The period of time may further include from about 20 minutes before surgery to about 1 hour after surgery. The period of time sufficient to treat an ischemic disorder in a subject who is not undergoing surgery may include before and during any physical manifestation of such disorder. Administration of carbon monoxide is preferable before

the manifestation in order to lessen such manifestation of an ischemic disorder. Carbon monoxide administration has been shown as described hereinbelow to be protective of ischemia in a subject if administered prior to surgery.

As used herein, the "ischemic disorder" encompasses and is not limited to a peripheral vascular disorder, a venous thrombosis, a pulmonary embolus, a myocardial infarction, a transient ischemic attack, lung ischemia, unstable angina, a reversible ischemic neurological deficit, adjunct thromolytic activity, excessive clotting conditions, sickle cell anemia or a stroke disorder.

The subject may be undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery. The organ transplantation surgery may include heart, lung, pancreas or liver transplantation surgery.

The carbon monoxide may be administered in an indirect manner. Rather than the subject directly inhaling or receiving carbon monoxide gas or a gas mixture, the subject may be given compunds to stimulate the in vivo production of carbon monoxide. Such compounds may include but are not limited to heme, ferritin, hematin, endogenous precursors to heme oxygenase or heme oxygenase stimulators. In addition, the subject may be exposed to an environment of low oxygen level compared to the normal atmosphere.

Heme oxygenase is an endogenous enzyme which synthesizes carbon monoxide from precursor heme (it is part of the normal way in which heme is degraded and metabolized in the body). When mice were exposed to either hypoxia or tissue ischemia, levels of both the messenger RNA which codes for heme oxygenase protein and the protein itself were increased. In addition, the activity of the enzyme was increased, as indicated by measurements of carbon monoxide in the tissue.

Another embodiment of the present invention is a method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of inactivated Factor IX in a sufficient amount over a sufficient period of time to inhibit coagulation so as to treat the ischemic disorder in the subject. The sufficient amount may include but is not limited to from about 75 μg/kg to about 550 μg/kg. The amount may be 300 μg/kg. The pharmaceutically acceptable form of inactivated Factor IX includes inactivated Factor IX and a pharmaceutically acceptable carrier.

The Factor IX may be inactivated by the standard methods known to one of skill in the art, such as heat inactivation. Factor IX may be inactivated or Factor IX activity may be inhibited by an antagonist. Such antagonist may be a peptide mimetic, a nucleic acid molecule, a ribozyme, a polypeptide, a small molecule, a carbohydrate molecule, a monosaccharide, an oligosaccharide or an antibody.

The present invention provides for a method for identifying a compound that is capable of improving an ischemic disorder in a subject which includes: a) administering the compound to an animal, which animal is a stroke animal model; b) measuring stroke outcome in the animal, and c) comparing the stroke outcome in step (b) with that of the stroke animal model in the absence of the compound so as to identify a compound capable of improving an ischemic disorder in a subject. The stroke animal model includes a murine model of focal cerebral ischemia and reperfusion. The stroke outcome may be measured by physical examination, magnetic resonance imaging, laser doppler flowmetry, triphenyl tetrazolium chloride staining, chemical assessment of neurological deficit, computed tomography scan, or cerebral cortical blood flow. The stroke outcome in a human may be measured also by clinical measurements, quality of life scores and neuropsychometric testing. The compound may include a P-selectin antagonist, an E-selectin antagonist or an L-

selectin antagonist.

The present invention further provides a method for identifying a compound that is capable of preventing the accumulation of white blood cells in a subject which includes:a) administering the compound to an animal, which animal is a stroke animal model; b) measuring stroke outcome in the animal, and c) comparing the stroke outcome in step (b) with that of the stroke animal model in the absence of the compound so as to identify a compound capable of preventing the accumulation of white blood cells in the subject.

The white blood cell may be, but is not limited to, a neutrophil, a platlet or a monocyte. The compound may be but is not limited to a selectin inhibitor, a monocyte inhibitor, a platelet inhibitor or a neutrophil inhibitor. The selectin inhibitor may be but is not limited to a P-selectin, E-selectin or L-selectin inhibitor. Selectins are expressed on the surface of platlets and such selectin inhibitors or antagonists as described herein may prevent the expression of such selectins on the surface of the cell. The prevention of expression may be at the transcriptional, translational, post-translational levels or preventing the movement of such selectins through the cytosol and preventing delivery at the cell surface.

The present invention provides for treatment of ischemic disorders by inhibiting the ability of the neutrophil, monocyte or other white blood cell to adhere properly. This may be accomplished removing the counter ligand, such as CD18. It has been demonstrated as discussed hereinbelow, that "knock-out" CD18 mice (mice that do not have expression of the normal CD18 gene) are protected from adverse ischemic conditions. The endothelial cells on the surface of the vessels in the subject may also be a target for treatment. In a mouse model of stroke, administration of TPA as a thrombolytic agent caused some visible hemorrhaging along with improvement of the stroke disorder. However, administration of a

P-selectin antagonist also improved stroke disorder in the animal model, but without the coincident hemorrhaging. The present invention may be used in conjunction with a thrombolytic therapy to increase efficacy of such therapy or to enable lower doses of such therapy to be administered to the subject so as to reduce side effects of the thrombolytic therapy.

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

This invention also provides for pharmaceutical compositions including therapeutically effective amounts of protein compositions and compounds capable of treating stroke disorder or improving stroke outcome in the subject of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in treatment of neuronal degradation due to aging, a learning disability, or a neurological disorder. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent

absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition. The choice of compositions will depend on the physical and chemical properties of the compound capable of alleviating the symptoms of the stroke disorder or improving the stroke outcome in the subject.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Portions of the compound of the invention may be "labeled" by association with a detectable marker substance (e.g., radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detection and quantification of compound or its receptor bearing cells or

its derivatives in solid tissue and fluid samples such as blood, cerebral spinal fluid or urine.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The compound of the present invention capable of

alleviating symptoms of a cognitive disorder of memory or learning may be delivered in a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

By means of well-known techniques such as titration and by taking into account the observed pharmacokinetic characteristics of the agent in the individual subject, one of skill in the art can determine an appropriate dosing regimen. See, for example, Benet, et al., "Clinical Pharmacokinetics" in ch. 1 (pp. 20-32) of Goodman

and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, A.G. Gilman, et al. eds. (Pergamon, New York 1990).

The present invention provides for a pharmaceutical composition which comprises an agent capable of treating a stroke disorder or improving stroke outcome and a pharmaceutically acceptable carrier. The carrier may include but is not limited to a diluent, an aerosol, a topical carrier, an aquous solution, a nonaqueous solution or a solid carrier.

This invention is illustrated in the Experimental Detail section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Abbreviations: EC, endothelial cell; PMN, polymorphonuclear leukocyte; WP, Weibel-Palade body; vWF, von Willebrand factor; EGTA, ethyleneglycol bis (aminoethylether)tetraacetic acid; HBSS, Hank's balanced salt solution; CS, coronary sinus; IL, interleukin; PAF, platelet activating factor; ICAM-1, intercellular adhesion molecule-1; HUVEC, human umbilical vein EC; LR, lactated Ringer's solution; MCAO, middle cerebral artery occlusion; rt-PA, recombinant tissue plasminogen activator; HO-I or HOI or HO I, heme oxygenase I.

EXAMPLE 1: Cerebral Protection in Homozygous Null ICAM-1 Mice Following Middle Cerebral Artery Occlusion: Role of Neutrophil Adhesion in the Pathogenesis of Stroke

To investigate whether polymorphonuclear leukocytes (PMNs) contribute to adverse neurologic sequelae and mortality following stroke, and to study the potential role of the leukocyte adhesion molecule Intercellular Adhesion Molecule-1 (ICAM-1) in the pathogenesis of stroke, a murine model of transient focal cerebral ischemia was employed consisting of intraluminal middle cerebral artery (MCA) occlusion for 45 minutes followed by 22 hours of reperfusion. PMN accumulation, monitored by deposition of ^{111}Indium-labelled PMNs in postischemic cerebral tissue, was increased 2.5-fold in the ipsilateral (infarcted) hemisphere compared with the contralateral (noninfarcted) hemisphere ($p < 0.01$). Mice immunodepleted of neutrophils prior to surgery demonstrated a 3.0-fold reduction in infarct volumes ($p < 0.001$), based on triphenyltetrazolium chloride staining of serial cerebral sections, improved ipsilateral cortical cerebral blood flow (measured by laser doppler) and reduced neurological deficit compared with controls. In wild type mice subjected to 45 minutes of ischemia followed by 22 hours of reperfusion, ICAM-1 mRNA was increased in the ipsilateral hemisphere, with immunohistochemistry

localizing increased ICAM-1 expression on cerebral microvascular endothelium. The role of ICAM-1 expression in stroke was investigated in homozygous null ICAM-1 mice (ICAM-1 -/-) in comparison with wild type controls (ICAM-1 +/+). ICAM-1 -/- mice demonstrated a 3.7-fold reduction in infarct volume ($p < 0.005$), a 35% increase in survival ($p < 0.05$), and reduced neurologic deficit compared with ICAM-1 +/+ controls. Cerebral blood flow to the infarcted hemisphere was 3.1-fold greater in ICAM-1 -/- mice compared with ICAM-1 +/+ controls ($p < 0.01$), suggesting an important role for ICAM-1 in the genesis of post-ischemic cerebral no-reflow. Because PMN-depleted and ICAM-1 deficient mice are relatively resistant to cerebral ischemia-reperfusion injury, these studies suggest an important role for ICAM-1-mediated PMN adhesion in the pathophysiology of evolving stroke.

INTRODUCTION:

Neutrophils (PMNs) are critically involved in the earliest stages of inflammation following tissue ischemia, initiating scavenger functions which are later subsumed by macrophages. There is a darker side to neutrophil influx, however, especially in postischemic tissues[1-7], where activated PMNs may augment damage to vascular and parenchymal cellular elements. Experimental evidence points to a pivotal role for endothelial cells in establishing postischemic PMN recruitment, in that hypoxic/ischemic endothelial cells synthesize the proinflammatory cytokine IL-1[8] as well as the potent neutrophil chemoattractant and activator IL-8[9]. Firm adhesion of PMNs to activated endothelium in a postischemic vascular milieu is promoted by translocation of P-selectin to the cell surface[10] as well as enhanced production of platelet activating factor and ICAM-1[11].

While strategies to block each of these mechanisms of neutrophil recruitment are protective in various models of ischemia and reperfusion injury, their effectiveness in cerebral ischemia/reperfusion injury remains controversial. There is

considerable evidence that in the brain, as in other tissues, an early PMN influx follows an ischemic episode[12-17]. Immunohistochemical studies have described increased expression of the PMN adhesion molecules P-selectin and intercellular adhesion molecule-1 (ICAM-1) in the postischemic cerebral vasculature[12,18-20]. The pathogenic relevance of adhesion molecule expression in the brain remains controversial, however; data from a trial of a monoclonal anti-ICAM-1 antibody in stroke in humans is not yet available. In animal models, there is conflicting experimental evidence regarding the effectiveness of anti-adhesion molecule strategies in the treatment of experimental stroke[21-23]. To determine whether ICAM-1 participates in the pathogenesis of postischemic cerebral injury, the experiments reported here were undertaken in a murine model of focal cerebral ischemia and reperfusion so that the role of a single, critical mediator of PMN adhesion (ICAM-1) could be determined. These studies demonstrate that enhanced ICAM-1 expression and neutrophil influx follow an episode of focal cerebral ischemia. Furthermore, these studies show that both neutrophil-deficient and transgenic ICAM-1 null mice are relatively resistant to cerebral infarction following ischemia and reperfusion, providing strong evidence for an exacerbating role of ICAM-1 in the pathophysiology of stroke.

MATERIALS AND METHODS

Mice: Experiments were performed with transgenic ICAM-1 deficient mice created as previously reported[24] by gene targeting in J1 embryonic stem cells, injected into C57BL/6 blastocysts to obtain germline transmission, and backcrossed to obtain homozygous null ICAM-1 mice. All experiments were performed with ICAM-1 -/- or wild-type (ICAM-1+/+) cousin mice from the fifth generation of backcrossings with C57BL/6 mice. Animals were seven to nine weeks of age and weighed between 25-36 grams at the time of experiments. For certain experiments, neutrophil depletion of C57BL/6 mice was accomplished by administering polyclonal rabbit anti-mouse neutrophil antibody[25] (Accurate Scientific, Westbury NY) preadsorbed

to red blood cells as a daily intraperitoneal injection (0.3 mL of 1:12 solution) for 3 days. Experiments in these mice were performed on the fourth day after confirming agranulocytosis by Wright-Giemsa stained peripheral blood smears.

Transient Middle Cerebral Artery Occlusion[26]: Mice were anesthetized with an intraperitoneal injection of 0.3 ml of ketamine (10 mg/cc) and xylazine (0.5 mg/cc). Animals were positioned supine on a rectal-temperature controlled operating surface (Yellow Springs Instruments, Inc., Yellow Springs, OH). Animal core temperature was maintained at 36-38°C intraoperatively and for 90 minutes post-operatively. Middle cerebral artery occlusion was performed as follows; A midline neck incision was created to expose the right carotid sheath under the operating microscope (16-25X zoom, Zeiss, Thornwood, NY). The common carotid artery was freed from its sheath, and isolated with a 4-0 silk, and the occipital and pterygopalatine arteries were each isolated and divided. Distal control of the internal carotid artery was obtained and the external carotid was cauterized and divided just proximal to its bifurcation into the lingual and maxillary divisions. Transient carotid occlusion was accomplished by advancing a 13 mm heat-blunted 5-0 nylon suture via the external carotid stump to the origin of the middle cerebral artery. After placement of the occluding suture, the external carotid artery stump was cauterized to prevent bleeding through the arteriotomy, and arterial flow was reestablished. In all cases, the duration of carotid occlusion was less than two minutes. After 45 minutes, the occluding suture was withdrawn to establish reperfusion. These procedures have been previously described in detail[26].

Measurement of cerebral cortical blood flow: Transcranial measurements of cerebral blood flow were made using laser doppler flow measurements (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as previously described[27] (transcranial readings were consistently the same as those made

after craniectomy in pilot studies). Using a 0.7 mm straight laser doppler probe (model #PF303, Perimed, Piscataway, NJ) and previously published landmarks (2 mm posterior to the bregma, 6 mm to each side of midline), relative cerebral blood flow measurements were made as indicated; immediately after anesthesia, after occlusion of the middle cerebral artery, immediately after reperfusion, and at 24 hours just prior to euthanasia. Data are expressed as the ratio of the doppler signal intensity of the ischemic compared with the nonischemic hemisphere. Although this method does not quantify cerebral blood flow per gram of tissue, use of laser doppler flow measurements at precisely defined anatomic landmarks serves as a means of comparing cerebral blood flows in the same animal serially over time. The surgical procedure/intraluminal middle cerebral artery occlusion and reperfusion were considered to be technically adequate if ≥ 50% reduction in relative cerebral blood flow was observed immediately following placement of the intraluminal occluding suture and a ≥ 33% increase in flow over baseline occlusion was observed immediately following removal of the occluding suture. These methods have been used in previous studies[26].

Preparation and administration of ^{111}In-labelled of murine neutrophils: Citrated blood from wild type mice was diluted 1:1 with NaCl (0.9%) followed by gradient ultracentrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Following hypotonic lysis of residual erythrocytes (20 sec exposure to distilled H_2O followed by reconstitution with 1.8% NaCl), the neutrophils were suspended in phosphate buffered saline (PBS). 5-7.5 x 10^6 neutrophils were suspended in PBS with 100 μCi of ^{111}Indium oxine (Amersham Mediphysics, Port Washington, NY) for 15 minutes at 37 °C. After washing with PBS, the neutrophils were gently pelleted (450 g), and resuspended in PBS to a final concentration of 1.0 x 10^6 cells/ml. Immediately prior to surgery, 100 μL of radiolabelled PMNs admixed with physiologic saline to a total volume of 0.3 mL (≈3 x 10^6 cpm) was administered by penile vein

injection. Following humane euthanasia, brains were obtained as described, and neutrophil deposition quantified as cpm/gm of each hemisphere.

Neurological Exam: Twenty-four hours after middle cerebral artery occlusion and reperfusion, prior to giving anesthesia, mice were examined for neurological deficit using a four-tiered grading system[26]: A score of (1) was given if the animal demonstrated normal spontaneous movements; a score of (2) was given if the animal was noted to be turning to the right (clockwise circles) when viewed from above (i.e., towards the contralateral side); a score of (3) was given if the animal was observed to spin longitudinally (clockwise when viewed from the tail); a score of (4) was given if the animal was crouched on all fours, unresponsive to noxious stimuli. This scoring system has been previously described in mice[26], and is based upon similar scoring systems used in rats[28,29] which are based upon the contralateral movement of animals with stroke; following cerebral infarction, the contralateral side is "weak" and so the animal tends to turn towards the weakened side. Previous work in rats[28] and mice[26] demonstrates that larger cerebral infarcts are associated with a greater degree of contralateral movement, up to the point where the infarcts are so large that the animal remains unresponsive.

Calculation of Infarct Volume: After neurologic examination, mice were given 0.3 mL of ketamine (10 mg/ml) and xylazine (0.5 mg/ml), and final cerebral blood flow measurements were obtained. Humane euthanasia was performed by decapitation under anesthesia, and brains were removed and placed in a mouse brain matrix (Activational Systems Inc., Warren, MI) for 1 mm sectioning. Sections were immersed in 2% 2,3,5, triphenyl, 2H-tetrazolium chloride (TTC, Sigma Chemical Co., St. Louis, MO) in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37°C, and placed in 10% formalin[26,30-32]. Infarcted brain was visualized as an area of unstained tissue, in contrast to viable tissue, which

stains brick red. Infarct volumes were calculated from planimetered serial sections and expressed as the percentage of infarct in the ipsilateral hemisphere.

RNA extraction and Northern blot analysis: 24 hours following focal ischemia and reperfusion, brains were obtained and divided into ipsilateral (infarct) and contralateral (noninfarct) hemispheres. To detect ICAM-1 transcripts, total RNA was extracted from each hemisphere using an RNA isolation kit (Stratagene, La Jolla, CA). Equal amounts of RNA (20 μg/lane) were loaded onto a 1.4% agarose gel containing 2.2 M formaldehyde for size fractionation, and then transferred overnight to nylon (Nytran) membranes with 10X SSC buffer by capillary pressure. A murine ICAM-1 cDNA probe[33] (1.90 kb, ATCC, Rockville, MD) was labelled with ^{32}P-α-dCTP by random primer labelling (Prime-A-Gene kit, Promega), hybridized to blots at 42°C, followed by 3 washes of 1X SSC/0.05% SDS. Blots were developed with X-Omat AR film exposed with light screens at -70° for 7 days. A β-actin probe (ATCC) was used to confirm equal RNA loading.

Immunohistochemistry: Brains were removed at the indicated times following middle cerebral artery occlusion, fixed in 10% formalin, paraffin embedded and sectioned for immunohistochemistry. Sections were stained with a rat anti-murine ICAM-1 antibody (1:50 dilution, Genzyme, Cambridge MA), and sites of primary antibody binding were visualized by an alkaline phosphatase conjugated secondary antibody detected with FastRed (TR/naphthol AS-MX, Sigma Chemical Co., St. Louis MO).

Data Analysis: Cerebral blood flow, infarct volumes and neurologic outcome scores were compared using Student's t-test for unpaired variables. ^{111}Indium-neutrophil deposition was evaluated as paired data [comparing contralateral (noninfarct) to ipsilateral (infarct) hemisphere], to control for variations in injected counts or volume of distribution. Survival differences between groups was tested

using contingency analysis with the Chi-square statistic. Values are expressed as means ± SEM, with a $p < 0.05$ considered statistically significant.

RESULTS:

Neutrophil Accumulation in Stroke: Previous pathologic studies have shown neutrophil accumulation following cerebral infarction[15-17,34-36]. To determine whether neutrophils accumulate in the murine model of focal cerebral ischemia and reperfusion, neutrophil accumulation following transient (45 min) ischemia and reperfusion (22 hrs) was quantified by measuring the deposition of ^{111}In-labeled neutrophils given to wild-type mice prior to the ischemic event. These experiments demonstrated significantly greater neutrophil accumulation (2.5-fold increase) in the ipsilateral (infarcted) compared with the contralateral (noninfarcted) hemispheres ($n = 7$, $p < 0.01$; **Figure 1**). Similar results were obtained when neutrophil influx was monitored by myeloperoxidase assays, though low levels of activity were recorded in the latter assay (data not shown).

Effect of Neutrophil Depletion on Stroke Outcome: To determine the effect of neutrophil influx on indices of stroke outcome, mice were immunodepleted of neutrophils beginning three days prior to surgery. When surgery was performed on the fourth day, nearly complete agranulocytosis was evident on smears of peripheral blood. Neutropenic mice ($n = 18$) were subjected to 45 min cerebral ischemia and 22 hours of reperfusion, and indices of stroke outcome determined. Infarct volumes were 3-fold smaller in neutropenic animals compared with wild type controls (11.1 ± 1.6% vs 33.1 ± 6.4%, $p < 0.001$; **Figure 2A**). The decrease in infarct volumes in neutropenic mice was paralleled by reduced neurologic deficit scores (**Figure 2B**), increased post-reperfusion cerebral cortical blood flows (**Figure 2C**), and a trend towards reduced overnight mortality (22% mortality in neutropenic mice vs 50% mortality in controls, **Figure 2D**).

ICAM-1 Expression in Murine Stroke: To establish the effect of cerebral ischemia/reperfusion in the murine model, ICAM-1 mRNA levels were evaluated following cerebral ischemia and reperfusion in wild type mice. Ipsilateral (infarcted) cerebral hemisphere demonstrated increased ICAM-1 mRNA by Northern blot analysis compared with RNA obtained from the contralateral (noninfarcted) hemisphere from the same animal (**Figure 3**). To evaluate ICAM-1 antigen expression in this murine model, wild type mice were subjected to 45 minutes of ischemia followed by 23 hours of reperfusion, and the cerebral microvasculature examined by immunohistochemistry. ICAM-1 antigen expression was not detectable in the cerebral microvasculature contralateral to the infarct (**Figure 4A**), but was markedly increased on the ipsilateral side, with prominent ICAM-1 staining of cerebral endothelial cells (**Figure 4B**).

Role of ICAM-1 in Stroke: To explore the role of ICAM-1 in stroke, transgenic mice which were homozygous ICAM-1 deficient[24] were studied in the murine model of focal cerebral ischemia and reperfusion. Because variations in cerebrovascular anatomy have been reported to result in differences in susceptibility to experimental stroke in mice[37], India ink staining was performed on the Circle of Willis in homozygous null (ICAM-1 -/-) and ICAM-1 +/+ mice. These experiments (**Figure 5**) demonstrated that there were no gross anatomic differences in the vascular pattern of the cerebral circulation. To determine the role of the intercellular adhesion molecule-1 in neutrophil influx following focal cerebral ischemia and reperfusion, neutrophil accumulation was measured in homozygous null ICAM-1 mice (ICAM-1 -/-) mice (n = 14) and wild-type controls (n=7) infused with ^{111}In-labeled neutrophils. Relative neutrophil accumulation (ipsilateral cpm/contralateral cpm) was diminished (39% reduction) in the ICAM-1 -/- mice compared with ICAM-1 +/+ controls (1.70 ± 0.26 vs. 2.9 ± 0.52, $p < 0.05$).

Experiments were then performed to investigate whether expression

of ICAM-1 has a pathophysiologic role in outcome following stroke. ICAM-1 -/- mice (n = 13) were significantly protected from the effects of focal cerebral ischemia and reperfusion, based on a 3.7-fold reduction in infarct volume ($p < 0.01$) compared with ICAM-1 +/+ controls (**Figures 6 and 7A**). This reduction in infarct volume was accompanied by reduced neurologic deficit (**Figure 7B**) and increased post-reperfusion cerebral cortical blood flow (**Figure 7C**). Given these results, it was not surprising that mortality was also significantly decreased in the the ICAM-1 -/- mice compared with ICAM-1 +/+ controls (15% vs. 50%, $p < 0.05$; **Figure 7D**).

DISCUSSION:

Epidemiologic evidence in humans suggests that neutrophils contribute to both the initiation of stroke[38] as well as to cerebral tissue injury and poor clinical outcome[39], with a potential role for neutrophils in postischemic hypoperfusion, neuronal dysfunction, and scar formation[40-44]. Although there is considerable experimental evidence which suggests that neutrophils can exacerbate tissue damage following stroke[13,45-48], certain pieces of experimental data have stoked controversy by failing to find an association between agents which block neutrophil accumulation and indices of stroke outcome. In a rat model of stroke, antibody-mediated depletion of neutrophils prior to stroke significantly decreased brain water content and infarct size[13]. However, cyclophosphamide-induced leukocytopenia in a gerbil model[49] or anti-neutrophil antibody administration to dogs[50] showed no beneficial effects in global models of cerebral ischemia. Experimental therapy targeted at interfering with neutrophil-endothelial interactions has also produced mixed results. In a feline model of transient focal cerebral ischemia, treatment with antibody to CD18 (the common subunit of β_2 integrins, which bind to intercellular adhesion molecule-1[51]) did not alter recovery of cerebral blood flow, return of evoked potentials, or infarct volume[23]. Other experiments, however, have found that microvascular patency after transient focal ischemia in primates is improved by antibodies to CD18[14]. In

a similar rat model, anti-CD11b/CD18 antibody has also been shown to reduce both neutrophil accumulation and ischemia-related neuronal damage[52].

The experiments reported here show that in a murine model of focal cerebral ischemia and reperfusion, neutrophils accumulate in postischemic cerebral tissue, a finding corroborated in other models which similarly demonstrate increased granulocyte accumulation in areas of low cerebral blood flow early during the post-ischemic period[15,16,36,45]. Not only do neutrophils accumulate during the post-ischemic period in mice, but their presence exacerbates indices of stroke outcome. When animals were made neutropenic prior to the ischemic event, cerebral infarcts were smaller, with improved cerebral perfusion following the ischemic event. These data are quite similar to that reported in a rabbit model of thromboembolic stroke, in which immunodepletion of neutrophils resulted both in reduced infarction volume and improved blood flow[35]. Because neutrophils contribute to murine post-ischemic cerebral injury, a strategy was pursued to elucidate the role of ICAM-1 in the pathophysiology of stroke using deletionally mutant ICAM-1 mice[24]. Experiments indicate that homozygous null ICAM-1 mice are relatively resistant to the deleterious effects of cerebral ischemia and reperfusion.

To demonstrate the role of both neutrophils and ICAM-1 in the pathogenesis of tissue injury in stroke, the studies reported here used several methods for assessing stroke outcome. Although numerous investigators have used TTC staining to quantify cerebral infarct volumes[26,30-32,37,53], there has been some controversy as to the accuracy of this method, especially when evaluated early following the ischemic event. In the TTC method, 2,3,5 triphenyl, 2H-tetrazolium chloride (TTC) reacts with intact oxidative enzymes on mitochondrial cristae and is thereby reduced to a colored formazan[54]. TTC staining is unreliable before 2 hours of ischemia have elapsed; beyond 36 hours, cells infiltrating into the

infarcted tissue can stain positively with TTC, thereby obscuring the clear demarcation between infarcted and noninfarcted tissues seen with earlier staining[31]. Although the size of the infarct delineated by TTC staining correlates well with infarct size delineated by hematoxylin and eosin staining[30,32], direct morphometric measurements tend to overestimate infarct volumes due to cerebral edema, especially during the first 3 days following the ischemic event[32]. Even given these limitations, however, the studies reported here incorporate three additional methods to define the role of neutrophils and ICAM-1 in stroke outcome, including neurologic deficit score, relative cerebral blood flow to the affected area, and mortality. These additional measures, which do not depend upon the accuracy of TTC staining, contribute strongly to the identification of a pathogenic role for both neutrophils and ICAM-1 in stroke.

There has been a recent profusion of scientific studies exploring the mechanistic basis for neutrophil recruitment to post-ischemic tissues. Endothelial cells appear to be the chief regulators of neutrophil traffic, regulating the processes of neutrophil chemoattraction, adhesion, and emigration from the vasculature[55]. When exposed to a hypoxic environment as a paradigm for tissue ischemia, endothelial cells synthesize the potent neutrophil chemoattractant and activator Interleukin-8 (IL-8)[9], the blockade of which appears to be beneficial in a lung model of ischemia and reperfusion[6]. In addition, hypoxic endothelial cells synthesize the proinflammatory cytokine Interleukin-1[8], which can upregulate endothelial expression of the neutrophil adhesion molecules E-selectin and ICAM-1 in an autocrine fashion[8,9,56]. Other neutrophil adhesion mechanisms may also be activated in the brain following ischemia, such as release of P-selectin from preformed storage pools within Weibel-Palade body membranes[10]. In a primate model, P-selectin expression was rapidly and persistently enhanced following focal middle cerebral artery ischemia and reperfusion[18]. Although P-selectin-dependent neutrophil recruitment appears to be

deleterious following cardiac ischemia and reperfusion[57], its pathophysiologic relevance in the setting of stroke has not yet been determined. While hypoxia induces *de novo* synthesis of the bioactive lipid platelet activating factor (PAF)[11], in a spinal cord ischemia reperfusion model, PAF antagonism offered no incremental benefit when given simultaneously with antibody to CD11/CD18[48].

Understanding the role of ICAM-1 in the pathophysiology of stroke appears to be of particular relevance in humans for several reasons. Increased cerebrovascular ICAM-1 expression has been demonstrated in primates by 4 hours of ischemia and reperfusion, particularly in the lenticulostriate microvasculature[18]. An autopsy study of recent cerebral infarcts in humans also demonstrated increased ICAM-1 expression[20]. As rats also express cerebral vascular ICAM-1 within 24 hours in both a photochemically-induced model of rat cerebral ischemia[19] as well as a middle cerebral artery occlusion model[12], these data suggested the potential usefulness of transgenic ICAM-1 deficient mice in elucidating the pathophysiologic significance of increased post-cerebral ischemic ICAM-1 expression. In particular, the time frame of ICAM-1 expression (increased by 4 to 24 hours) in these models suggests that ICAM-1 mediated neutrophil-endothelial interactions may be targeted in future pharmacologic strategies to improve human stroke outcome, as this time frame represents a realistic clinical window for therapeutic intervention.

Although neutropenic animals demonstrated increased regional cerebral blood flow compared with controls, compared with neutropenic animals, ICAM-1 deficient mice tended to have even higher ipsilateral cerebral blood flows at 24 hours. This observation may relate to the no-reflow phenomenon, wherein blood flow fails to return to pre-obstruction levels even following release of a temporary vascular occlusion. A significant body of previous work has implicated neutrophil plugging of capillary microvascular beds in this process[58], although in a model of global

cerebral ischemia, an 85% reduction in the circulating leukocyte count did not decrease the incidence or severity of reflow failure[49]. The data suggest that non-neutrophil-dependent mechanisms, which nevertheless involve ICAM-1, may contribute to cerebrovascular post-ischemic no-reflow. As macrophages and lymphocytes both express LFA-1, which mediates an adhesive interaction with endothelial cell ICAM-1[51], it is possible that ICAM-1 deficient mice have diminished recruitment of these mononuclear cells, a possibility which is currently the subject of further investigation. This hypothesis is supported by multiple pathologic observations demonstrating macrophage and lymphocyte accumulation by 1-3 days following cerebral infarction[12,17,19,34,59].

Taken together, the studies indicate that in a murine model of focal cerebral ischemia and reperfusion, neutrophils accumulate in the infarcted hemisphere, and that neutropenic animals demonstrate cerebral protection. Increased expression of ICAM-1 on cerebral endothelial cells appears to be an important mechanism driving this neutrophil recruitment, and mice which are unable to express ICAM-1 demonstrate improved post-ischemic blood flows, reduced infarction volumes, and reduced mortality. These data suggest that pharmacologic strategies targeted at interfering with neutrophil-endothelial interactions may improve the outcome following stroke in humans.

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EXAMPLE 2: Hypoxia-Induced Exocytosis of Endothelial Cell Weibel-Palade Bodies: A Mechanism for Rapid Neutrophil Recruitment Following Cardiac Preservation

The period of hypoxia (H) is an important priming event for the vascular dysfunction which accompanies reperfusion, with endothelial cells (ECs) and neutrophils (PMNs) playing a central role. It was hypothesized that EC Weibel-Palade (WP) body exocytosis during the hypoxic/ischemic period during organ preservation permits brisk PMN recruitment into post-ischemic tissue, a process further amplified in an oxidant-rich mileu. Exposure of human umbilical vein ECs to an hypoxic environment (pO_2 ≈20 torr) stimulated release of von Willebrand factor (vWF), stored in EC WP bodies, as well as increased expression of the WP body-derived PMN-adhesion molecule P-selectin at the EC surface. Increased binding of ^{111}In-labelled PMNs to hypoxic EC monolayers (compared with normoxic controls) was blocked with a blocking antibody to P-selectin, but was not effected by a nonblocking control antibody. Although increased P-selectin expression and vWF release were also noted during reoxygenation, H alone (even in the presence of antioxidants) was sufficient to increase WP body exocytosis. To determine the relevance of these observations to hypothermic cardiac preservation, during which the pO_2 within the cardiac vasculature declines to similarly low levels, experiments were performed in a rodent (rat and mouse) cardiac preservation/transplantation model. Immunodepletion of recipient PMNs or administration of a blocking anti-P-selectin antibody prior to transplantation resulted in reduced graft neutrophil infiltration and improved graft survival, compared with identically preserved hearts transplanted into control recipients. To establish the important role of endothelial P-selectin expression on the donor vasculature, murine cardiac transplants were performed

using homozygous P-selectin deficient and wild type control donor hearts flushed free of blood/platelets prior to preservation/transplantation. P-selectin null hearts transplanted into wild-type recipients demonstrated a marked (13-fold) reduction in graft neutrophil infiltration and increased graft survival compared with wild type hearts transplanted into wild type recipients. To determine whether coronary endothelial Weibel-Palade body exocytosis may occur during cardiac preservation in humans, the release of vWF into the coronary sinus was measured in 32 patients during open heart surgery. Coronary sinus samples obtained at the start and conclusion of the ischemic period demonstrated an increase in coronary sinus vWF antigen (by ELISA) consisting of predominantly high molecular weight multimers (by immunoelectrophoresis). These data suggest that EC Weibel-Palade body exocytosis occurs during hypothermic cardiac preservation, priming the vasculature to rapidly recruit PMNs during reperfusion.

Introduction:

Endothelial cells (EC) adapt to hypoxia with a characteristic repertoire of responses (1), ranging from increased expression of endothelin (2) to increased synthesis of basic fibroblast growth factor (3). Recent studies have indicated that many features of the EC response to hypoxia parallel features of the inflammatory response; hypoxia selectively upregulates EC expression of Interleukins- 1 (4), 6 (5), and 8 (6), platelet activating factor (PAF) (7,8), and ICAM-1 (4), which serve to fuel neutrophil (PMN) recruitment, adhesion, and activation at ischemic loci. Although these mechanisms may explain the later phases of reperfusion injury, the rapidity with which PMNs are recruited to reperfused myocardium following a period of hypothermic preservation suggests that mechanisms are involved which do not require *de novo* protein synthesis. In this regard, P-selectin may figure prominently in the earliest phases of PMN adhesion to the reperfused vasculature, as ECs may rapidly express pre-formed P-selectin from

subplasmalemmal storage sites in Weibel-Palade body (9) membranes in response to the abundant oxygen free-radicals generated in the reperfusion milieu (10-12). Furthermore, recent data have pointed to a role for P-selectin-mediated leukocyte arrest in leukostasis and tissue damage associated with lung injury (13) and cardiac ischemia (14). Taken together, these findings led to the hypothesis that the hypoxic/ischemic period associated with hypothermic myocardial preservation primes the vasculature for its characteristic reponse during reperfusion by displaying P-selectin prominently at the EC surface prior to reperfusion, serving as a spark which ignites and amplifies the subsequent inflammatory response.

The experiments were designed to establish whether hypoxia *per se* (or hypothermic cardiac preservation, as occurs during cardiac surgery, in which the pO_2 within the coronary bed declines to pO_2<20 Torr) (15) would result in WP body exocytosis. Furthermore, experiments were undertaken to determine the role of P-selectin-dependent PMN adhesion in the cardiac graft failure which characteristically follows a period of prolonged hypothermic preservation. The results show that hypoxia is sufficient to induce EC WP body exocytosis, even in the absence of reoxygenation (and presence of antioxidants), and that the resulting P-selectin expression causes ECs to bind PMNs *in vitro*. In rodents, the adverse consequences of P-selectin expression following hypothermic cardiac preservation can be completely abrogated by either neutrophil depletion, P-selectin blockade, or by transplanting hearts whose endothelial cells fail to express P-selectin. Because WP body exocytosis also occurs in patients undergoing open heart surgery during the period of hypothermic cardiac preservation, these data suggest that P-selectin blockade may represent a target for pharmacological intervention to improve cardiac preservation in humans.

Methods:

Endothelial cell culture and exposure of cells to H or H/R. Human umbilical vein ECs were prepared from umbilical cords and grown in culture by the method of Jaffe (16) as modified by Thornton (17). Experiments utilized confluent ECs (passages 1-4) grown in Medium 199 supplemented with fetal bovine serum (15%; Gemini, Calabasas, CA), human serum (5%; Gemini), endothelial growth supplement (Sigma, St. Louis, MO), heparin (90 μg/ml; Sigma) and antibiotics, as described (17). When ECs achieved confluence, experiments were performed by placing cultures in an environmental chamber (Coy Laboratory Products, Ann Arbor, MI) which provided a controlled temperature (37°C) and atmosphere with the indicated amount of oxygen, carbon dioxide (5%) and the balance made up of nitrogen. Use of this chamber for cell culture experiments has been described previously (15,18). During exposure of ECs to hypoxia (for a maximum of 16 hours), the oxygen tension in the culture medium was 14-18 torr and there was no change in the medium pH. Reoxygenation was performed by placing ECs in an ambient atmosphere containing carbon dioxide (5%) at 37°C.

Measurement of Weibel-Palade body exocytosis: ECs were plated into 24 well plates, rinsed 3 times with Hank's balanced salt solution, and then exposed to hypoxia or to normoxia for the indicated durations. For experiments in which vWF was measured, cells were maintained in serum-free medium. All other EC experiments were performed in the EC growth medium described above. For measurement of vWF, 200 μL aliquots of culture supernatant was removed at the indicated times, and a commercially available ELISA (American Diagnostica, Greenwich, CT), based on a polyclonal goat anti-human vWF antibody, was performed on duplicate specimens, with a standard curve generated using purified human vWF antigen supplied by the same vendor. EC P-selectin expression was determined by measuring the specific binding of a murine monoclonal anti-human P-selectin antibody (WAPS 12.2 clone, Endogen, Cambridge, MA; this is an IgG1

which recognizes a calcium sensitive epitope and blocks P-selectin dependent neutrophil adhesion. Antibody was radiolabelled with ^{125}I by the lactoperoxidase method (19) using Enzymobeads (Bio-Rad, Hercules, CA), stored at 4 °C and used within one week of labelling. Binding assays were performed on HUVECs plated on 96 well plates, in which fresh M199 with 0.1% bovine serum albumin (Sigma, St. Louis, MO) was added immediately prior to each experiment. Cells were placed in a humidified environment at 37 °C, and exposed to normoxia or H (in the presence or absence of 50 μM probucol, as indicated, Sigma) for the indicated durations. Cell monolayers were fixed for 15 min. with 1% paraformaldehyde[10] (cells exposed to H were fixed while still within the hypoxic environment), visually inspected to ensure that the monolayers remained intact, and washed twice with HBSS containing 0.5% bovine serum albumin (HBSS/A). Monolayers were then exposed to 10^5 cpm of ^{125}I-labelled anti-P-selectin antibody (WAPS 12.2) in the presence of 200 μg/mL of either unlabelled blocking antibody (WAPS 12.2) or nonblocking anti-P-selectin IgG of the same isotype (anti-GMP-140, AC1.2 clone, Becton-Dickinson, San Jose, CA) (20,21). After binding for 1 hour at 37 °C, monolayers were washed 4 times with HBSS/A, and bound antibody was eluted with 1% triton X-100 in PBS (200 μL/well) and counted. For certain experiments, cycloheximide (10 μg/mL, Sigma) was added at the start of the 4 hour normoxic or hypoxic period, as indicated. In separate experiments, designed to determine the degree of inhibition of protein synthesis by cycloheximide treatment, ECs were incubated with methionine- and cysteine-poor minimal essential medium (Gibco, Grand Island, NY) in the presence of ^{35}S-methionine and ^{35}S-cysteine (either in the presence or absence of cyloheximide, 10 μg/mL) (3). Following 4 hours of normoxic exposure, trichloroacetic acid-precipitable material was collected and counted.

Preparation of human PMNs and Measurement of Binding: In brief, citrated blood from healthy donors was diluted 1:1 with NaCl (0.9%) followed by gradient ultracentrifugation on Ficoll-Hypaque

(Pharmacia, Piscataway, NJ). Following hypotonic lysis of residual erythrocytes (20 sec exposure to distilled H_2O followed by reconstitution with 1.8% NaCl), PMNs were suspended in HBSS with 5 mg/mL of human serum albumin (HBSS/HSA). 50-200 x 10^6 PMNs were suspended in HBSS/HSA in the presence of 0.2-0.5 μCi of ^{111}Indium oxine (Amersham Mediphysics, Port Washington, NY) for 15 minutes at 37 °C. After washing with HBSS/HSA, PMNs were gently pelleted (450 g), and resuspended in HBSS/HSA to a final concentration of 5.5 x 10^6 PMNs/mL. Following gentle agitation, 100 μL of the radiolabelled PMN suspension was added to each well at the indicated time, incubated for 30 minutes at 37 °C, and then washed 4 times with HBSS/HSA. Monolayers were then treated with 1 N NaOH and the contents of each well withdrawn and counted.

Heterotopic Rat and Mouse Cardiac Transplant Model. Cardiac transplants were performed in the Ono-Lindsey heterotopic isograft model of cardiac transplantation (15,18,22). Briefly, male Lewis rats (250-300 grams, Harlan Sprague Dawley, Indianapolis, IN) were anesthetized, heparinized, and the donor heart rapidly harvested following hypothermic high potassium cardioplegic arrest. Hearts were preserved by flushing the coronary arteries with 4°C lactated Ringer's (LR) solution (Baxter, Edison, NJ), sixteen hours of immersion in the same solution at 4°C, followed by heterotopic transplantation into gender/strain matched recipients, with sequential donor and recipient aortic and donor pulmonary arterial/recipient inferior vena caval anastomoses performed. Graft survival was assessed by the presence/absence of cardiac electrical/mechanical activity exactly ten minutes following reestablishment of blood flow, after which the graft was excised and neutrophil infiltration was quantified by myeloperoxidase activity, measured as previously described (15,18). For certain experiments, neutrophil depletion of recipient rats was accomplished by administering a polyclonal rabbit anti-rat neutrophil antibody (23-25) (Accurate Scientific, Westbury NY) as a single intravenous injection 24 hours prior to the

transplantation procedure. Neutrophil depletion in these animals was confirmed and quantified by counting remaining neutrophils, identified on Wright-Giemsa stained smears of peripheral blood. In other experiments, a blocking anti-P-selectin IgG (250 μg/rat, Cytel, San Diego, CA) (13,14,26) was administered intravenously 10 minutes prior to the onset of reperfusion. Murine heart transplants were performed in an identical fashion using homozygous P-selectin null or wild-type control male mice with a C57BL/6J background (27), with the harvested hearts immediately flushed free of native blood with 1.0 mL of 4°C LR administered down a cross-clamped aortic root followed by period of hypothermic preservation consisting of three hours of immersion in lactated Ringer's solution at 4°C.

Measurement of vWF in Coronary Effluent from Hypothermically Preserved Rat and Human Hearts:

Human coronary sinus samples. After obtaining informed consent, coronary sinus blood was obtained at the start and conclusion of routine cardiac surgery in an unselected series of 32 patients, with simultaneous sampling of peripheral (arterial) blood in six. Coronary sinus samples were obtained from a retrogade perfusion catheter which was routinely placed in patients undergoing cardiopulmonary bypass. Plasma samples were centrifuged for 5 min at 1500 x g to sediment cellular elements, and the plasma aliquoted and frozen at -70°C until the time of assay. ELISAs were performed for vWF (as described above) and thrombomodulin (Asserchron Thrombomodulin, Diagnostica Stago).

vWF immunoelectrophoresis: Multimeric composition of the vWF in coronary sinus plasma samples and endothelial cell supernatants was evaluated by performing agarose gel immunoelectrophoresis. Samples were diluted 1:10, 1:20, and 1:30 (as indicated) and incubated for 30 minutes at 37° C in Native Sample Buffer (Bio-Rad). Samples (20 μL) were then electrophoresed in a 1.5% agarose gel (0.675 g Low M^r agarose, Bio-Rad; 0.045 g SDS; 45 mL Tris-Tricine SDS Buffer

[Bio-Rad]). Molecular weight markers run simultaneously on agarose gels were visualized by marking and dividing the gel, with molecular weight marker locations assigned by Coomasie blue staining. The remaining half of the gel was washed in sodium borate (0.01 M) for 30 minutes followed by overnight electrophoretic transfer to a nitrocellulose membrane. The membrane was washed with washing buffer consisting of tris-buffered saline (pH 7.5) with 0.05% Tween-20, and then blocked for 1 hour with 50 mL of washing buffer containing 2.5 g of Carnation instant milk. After rinsing with physiologic saline, the membrane was immersed overnight in washing buffer containing 1g/dL gelatin and a 1:500 dilution of rabbit anti-human vWF serum (American Bioproducts, Parsippany, NJ). After washing 5 times with washing buffer, the membrane was immersed for 3 hours with gentle shaking in washing buffer containing 1g/dL gelatin and 16.6 μL of goat anti-rabbit horseradish peroxidase conjugated IgG (Bio-Rad), and developed with 60 mL of HRP Developer (30 mg HRP Developer powder, Bio-Rad; 10 mL methanol; 50 mL tris-buffered saline; 50 μL of 30% hydrogen peroxide added just prior to use).

Statistics. Analysis of variance was used to compare 3 or more conditions, with post-hoc comparisons tested using Tukey's procedure. Graft survival data was analyzed using contingency analysis with the Chi-square statistic. Paired comparison of serial measurements (human CS and peripheral blood samples at the start and conclusion of cardiac surgery) were compared using Student's t-test for paired variables. Values are expressed as means $\pm$ SEM, with a $p < 0.05$ considered statistically significant.

Results:

Exposure of cultured ECs to hypoxia results in the release of vWF and translocation of P-selectin to the cell surface. Previous studies have shown that exposure of endothelial cells to hypoxia results in an elevation in intracellular calcium (28). In view of

the association of increased cytosolic calcium with EC Weibel-Palade body exocytosis in response to thrombin or histamine (29,30), it was considered whether exposure of ECs to hypoxia could initiate this process. ECs placed in an hypoxic environment (pO_2 20 torr) released more vWF into the culture supernatants than their normoxic counterparts (**Fig. 8A,** ELISA; confirmed by immunoelectrophoresis, data not shown). Although a trend towards enhanced levels of vWF was first noted by 1 hour of hypoxia, the differences between normoxic and hypoxic vWF levels did not become statistically significant until 4 hours of exposure, thereafter increasing steadily for up to 12 hours of observation. To determine whether the increased vWF release seen by 4 hours of hypoxia was due to release of pre-formed vWF, similar experiments were performed in the presence of 10 μg/ml cycloheximide to inhibit protein synthesis. These experiments showed that addition of cycloheximide at the start of the hypoxic period decreased hypoxia-induced vWF release by 12.5%, suggesting that the majority of vWF released by hypoxic exposure was pre-formed.

Although these experiments were done in their entirety within the hypoxic environment (i.e., there was no reoxygenation), to further demonstrate that this H-mediated exocytosis of Weibel-Palade bodies was independent of the formation of reactive oxygen intermediates, the antioxidant probucol (50 μM) was added to the ECs at the onset of H and was found to have no effect (vWF $4.7 \pm 0.31 \times 10^{-3}$ U/ml at 6 hours of H). The presence of probucol did blunt the further increase in vWF levels seen following reoxygenation of the hypoxic ECs. The calcium-dependence of hypoxia-induced Weibel-Palade body exocytosis was demonstrated by experiments in which ECs were placed in a calcium-free medium at the start of hypoxic exposure. Absence of extracellular calcium attenuated H-induced EC release of vWF, and addition of EGTA had an even more suppressive effect (basal endothelial release of vWF was also diminished by the reduction of extracellular calcium) **(Fig. 8B)**.

To determine whether hypoxia also induced translocation of P-selectin to the EC plasmalemmal surface, specific binding of ^{125}I-labelled anti-P selectin IgG to normoxic or hypoxic EC monolayers was examined. Binding studies were performed on EC monolayers fixed with paraformaldehyde while still within the hypoxic environment, to obviate oxygen-free radical-induced P-selectin expression during reoxygenation. These studies demonstrated enhanced binding of ^{125}I-anti-P-selectin IgG by hypoxic compared with normoxic ECs **(Fig. 9A)**. This binding was blocked by unlabelled blocking anti-P-selectin IgG, but not by a nonblocking control anti-P-selectin IgG of the same isotype. Surface expression of P-selectin was noted at the earliest time points observed (60 minutes of H), and was observed at similar levels throughout the period of hypoxic exposure (up to 4 hours of observation). It is possible that hypoxia-induced endothelial P-selectin expression was detected at time points preceding a statistically significant increase of vWF release in similarly treated cells, because a portion of the initially secreted vWF binds tightly to subendothelial matrix (31).

To determine whether protein synthesis was required for hypoxia-induced P-selectin expression, a separate experiment was performed in which cyloheximide was given at the onset of normoxia or H, and binding of radiolabelled anti-P-selectin IgG determined at the 4 hour time point. This experiment demonstrated that even with > 85% inhibition of protein synthesis **(Fig. 9B, Inset)**, hypoxia still increased endothelial P-selectin expression, albeit at reduced levels **(Fig. 9B)**. To establish that hypoxia-induced cell-surface P-selectin may participate in neutrophil binding, human neutrophils radiolabelled with ^{111}indium oxine were incubated with hypoxic ECs; enhanced binding to hypoxic monolayers was observed. Hypoxia-induced ^{111}In-PMN binding was blocked by the addition of a blocking anti-P-selectin IgG, but not by a nonblocking anti-P-selectin IgG **(Fig. 9C)**.

Role of P-selectin dependent neutrophil adhesion in hypothermic/ischemic myocardial preservation. To establish the relevance of these observations to hypothermic myocardial preservation (in which the pO_2 of the preservation solution within the coronary vasculature drops below 20 Torr (15)), hearts were harvested from male Lewis rats and subjected to hypothermic preservation as described in the methods section. Because neutrophil-mediated damage following cardiac ischemia is well established (32-38), the potential pathophysiologic role of endothelial P-selectin expression was investigated in an orthotopic rat heart transplant model in which reperfusion occurred following a period of hypothermic preservation. These experiments showed excellent graft survival and little neutrophil infiltration if heart transplantation was performed immediately following harvest **(Fig. 10A, Fresh)**. However, when similar experiments were performed with an intervening (16 hour) period of hypothermic preservation between the harvest and transplantation procedures, there was a high incidence (90%) of graft failure and marked leukostasis, confirmed histologically and by determining myeloperoxidase activity **(Fig. 10A, Prsvd)**. To demonstrate that neutrophil adhesion was responsible, at least in part, for graft failure following prolonged preservation, transplants were performed following neutrophil depletion of recipient rats. The polyclonal rabbit anti-rat PMN antibody used (23-25) eliminated virtually all circulating PMNs in the recipients (PMN count 1471 ± 56 vs 67 ± 11 PMNs/mm^3 for control and immunodepleted animals, respectively, $p < 0.001$), with little effect on other cell types. When 16 hour preserved hearts were transplanted into neutrophil-depleted recipients to provide a neutrophil-free reperfusion milieu, there was a significant reduction in graft myeloperoxidase activity and an increase in graft survival **(Fig. 10A, Prsvd (-) PMN)**. Normal recipient rats infused with blocking anti-P-selectin IgG 10 minutes prior to reestablishment of blood flow demonstrated a reduction of both myeloperoxidase activity as well as improvement in graft

survival **(Fig. 10A, α-PS, Blocking)** of a similar magnitude as neutrophil-depleted recipients. This reduced PMN infiltration and improved graft survival was observed despite 16 hours of hypothermic preservation of the donor heart. In sharp contrast, administration of a nonblocking control antibody (AC1.2) had no beneficial effect on graft leukostasis or graft survival **(Fig. 10A, α-PS, Non-blocking)**.

Because in addition to the interactions between ECs and PMNs, platelets may also interact with PMNs via a P-selectin-dependent mechanism (39), an experiment was designed to isolate the contribution of endothelial P-selectin to the leukostasis and graft failure which occur following prolonged hypothermic cardiac preservation. For these experiments, donor hearts from homozygous P-selectin deficient mice could be flushed free of blood, so that P-selectin null coronary endothelial cells could be transplanted into wild type recipients with P-selectin containing platelets. Using a murine heterotopic heart transplant model performed identically to the rat operation, donor hearts were obtained from either homozygous P-selectin null mice (27) or wild-type controls; all hearts were transplanted into wild-type recipients. These experiments demonstrated a significantly higher graft survival rate in the P-selectin null → wild type transplants compared with wild type → wild type transplants **(Fig. 10B)**. This improved graft survival in the former group was paralled by a marked (13-fold) reduction in graft leukostasis **(Fig. 10C)**. Because these hearts had been flushed free of blood at the start of preservation, these studies implicate coronary endothelial (rather than platelet-derived) P-selectin in the poor preservation and leukocyte arrest noted after hypothermic myocardial preservation.

<u>Weibel-Palade body exocytosis during human cardiac surgery</u>. To establish the relevance of these findings to humans, the next set of experiments were designed to demonstrate that coronary ECs

release the contents of Weibel-Palade bodies during hypothermic cardiac preservation as occurs during routine cardiac surgery. Measurements were made of vWF release from the coronary vasculature during a well-defined period of cardiac ischemia, that which occurs during the period of aortic cross-clamping. Coronary sinus (draining the heart) blood was sampled at the start (CS_1) and conclusion (CS_2) of aortic cross clamping in 32 patients (this interval represents the ischemic period). These patients (23 male, 9 female) had a clinical history of valvular heart disease (n=11) or ischemic heart disease (n=21), and underwent either valve repair/replacement or coronary artery bypass grafting, respectively. Capture ELISAs performed for the integral membrane protein thrombomodulin (40) demonstrated no change in levels between the CS_1 and the CS_2 samples (4.35 ± 1.2 ng/mL vs 3.48 ± 0.8 ng/mL, p=NS), suggesting that ECs were not sloughed and cell membrane integrity was maintained during cardiac preservation. Similar measurements performed for vWF showed that there was a consistent and significant increase in vWF that is secreted during the course of cardiac preservation (0.68 ± 0.06 U/ml vs 0.90 ± 0.05 U/ml, CS_1 vs CS_2, $p<0.01$) **(Fig. 11A)**.

To demonstrate that this vWF was likely to be of coronary endothelial rather than of platelet origin, and hence not simply a consequence of cardiopulmonary bypass, peripheral blood samples were obtained simultaneously with the CS_1 and CS_2 samples, and showed that levels of vWF were unchanged (0.813 ± 0.52 U/mL vs 0.900 ± 0.41 U/mL, p=NS), suggesting that mechanical perturbation of platelets during cardiopulmonary bypass was not causative. Because vWF is present in plasma as multimers with a range of M_r's (41-44), with those vWF multimers from the stimulatable pool (as opposed to those constitutively secreted) being of the highest molecular weight (45), immunoelectrophoresis was performed on the CS samples. These gels demonstrated that in addition to an overall increase in vWF in the CS_2 samples, there appeared to be an increase in high molecular weight multimers, suggesting release

from a stimulatable pool, as is found in endothelial cells (**Fig. 11B**).

Discussion:

The vasculature plays a critical role in maintaining the extracellular milieu of organs subjected to ischemia and reperfusion, a role which is chiefly orchestrated by the ECs lining the endovascular lumen. The EC responds to a period of oxygen deprivation by striking phenotypic modulation, becoming prothrombotic (46) and proinflammatory (1,4,6). ECs exposed to hypoxia secrete the proinflammatory cytokines IL-1 (4) and IL-8 (6) which may serve to direct leukocyte traffic to areas of ischemia. Because these processes require *de novo* protein synthesis, they do not explain the immediate events which occur following a period of hypothermic preservation. While enhanced expression of ICAM-1 and induction of E-selectin may contribute at later times to leukocyte arrest in cardiac grafts, this does not explain the rapid leukostasis observed following cold preservation, in which protein synthesis is likely to be considerably slowed. In this context, cycloheximide pre-treatment does not alter the early (90-120 minute) PMN adhesion seen following hypoxic exposure of ECs (7), suggesting that *de novo* protein synthesis need not be involved in hypoxia-mediated increases in PMN binding. Although platelet activating factor (PAF) may participate in hypoxia-mediated PMN adhesion (7,47) and activation (48,49), PAF is not stored and must be synthesized, which may lessen its importance during the hypothermic period during myocardial preservation. It is for this reason that rapid EC expression of pre-formed P-selectin from subplasmalemmal storage sites in Weibel-Palade bodies (9,50,51) may represent the most important mechanism for early PMN recruitment following hypothermic preservation. Weibel-Palade bodies are found in abundance within the coronary microvasculature (52), suggesting their particular importance in cardiac preservation.

The data show Weibel-Palade exocytosis occurs both in response to

hypoxia *per se*, as well as in human hearts during hypothermic preservation. While it is difficult to precisely identify an endothelial origin for the vWF observed in the human coronary sinus samples, studies of platelets following cardiopulmonary bypass demonstrate no increase in surface P-selectin expression or α-granule secretion (53,54). This suggests that the observed increase in coronary sinus vWF following aortic cross-clamping is not of platelet origin. Two aspects of the data also suggest that the vWF released following ischemia is of endothelial origin; (1) Peripheral vWF levels remained unchanged while coronary sinus levels are increased following myocardial ischemia, suggesting that the elevated vWF was emanating from the heart, not the cardiopulmonary bypass apparatus; (2) The transgenic, P-selectin null donor hearts were flushed free of donor blood at the onset of preservation, so that when transplanted into wild-type recipients, presumably coronary endothelial (not platelet) P-selectin is absent. These experiments demonstrate the important contribution of *endothelial* P-selectin to the neutrophil recruitment which accompanies reperfusion.

It is not surprising that P-selectin should be important following hypothermic myocardial preservation; recent studies have demonstrated that P-selectin is an important mediator of neutrophil-induced reperfusion damage following normothermic ischemia, as has been shown in rabbit ear (26) and feline cardiac ischemia (14) models. Because oxidants cause expression of P-selectin at the EC surface (10), it was important in these studies to evaluate the role of the hypoxic period alone as it may prime ECs to recruit the first wave of PMNs, with further PMN recruitment amplified with the onslaught of reactive oxygen intermediates produced in the reperfusion microenvironment. Although one report has suggested that hypoxia might induce EC P-selectin expression, these experiments (7) were actually performed following reoxygenation, a condition which is known to induce both superoxide

(18,55) and neutrophil adherence to cultured ECs (56). By contrast, the experiments described herein were performed entirely within a hypoxic environment to completely prevent the possibility of reoxygenation, and antioxidants failed to block hypoxia-induced P-selectin expression, suggesting that the observations described herein reflect hypoxia hypoxia *per se* rather than reoxygenation. Furthermore, the cardiac protection demonstrated herein using a strategy whereby blood-free preserved hearts from transgenic P-selectin null mice are transplanted into recipients with wild-type platelets demonstrates that endothelial P-selectin expression can be deleterious following hypothermic cardiac preservation. Because Weibel-Palade body exocytosis occurs during hypothermic cardiac preservation in humans, these studies suggest that myocardial preservation may be enhanced by therapeutic strategies designed to block the activity of P-selectin expressed at the endothelial surface.

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EXAMPLE 3: Procedural and Strain-Related Variables Significantly Effect Outcome in a Murine Model of Focal Cerebral Ischemia

The recent availability of transgenic mice has led to a burgeoning number of reports describing the effects of specific gene products on the pathophysiology of stroke. Although focal cerebral ischemia models in rats have been well-described, descriptions of a murine model of middle cerebral artery occlusion are scant, and sources of potential experimental variability remain undefined. It was hypothesized that slight technical modifications would result in widely discrepant results in a murine model of stroke, and that controlling surgical and procedural conditions could lead to reproducible physiologic and anatomic stroke outcomes. To test this hypothesis, a murine model was established which would permit either permanent or transient focal cerebral ischemia by intraluminal occlusion of the middle cerebral artery (MCA). This study provides a detailed description of the surgical technique, and reveals important differences between strains commonly used in the production of transgenic mice. In addition to strain-related differences, infarct volume, neurologic outcome, and cerebral blood flow appear to be importantly affected by temperature during the ischemic and post-ischemic periods, mouse size, and size of the suture which obstructs the vascular lumen. When these variables were kept constant, there was remarkable uniformity of stroke outcome. These data emphasize the protective effects of hypothermia in stroke, and should help to standardize techniques among different laboratories to provide a cohesive framework for

evaluating the results of future studies in transgenic animals.

Introduction:

The recent advent of genetically altered mice provides a unique opportunity to evaluate the role of single gene products in the pathophysiology of stroke. Although there is an increasing number of reports about the effect of cerebral ischemia in transgenic mice, to date, there exists no detailed description of the murine models involved, nor is there a detailed analysis of potentially important procedural variables which may effect stroke outcome. Most descriptions of a murine model (1,4,8,9,14,17-19,23,24) are devolved descriptions of the widely used rat models of focal cerebral ischemia (22,26). Although there has been some attention paid to strain related differences in the susceptibility of mice to cerebral ischemia (4), few technical considerations have been addressed in published studies. Because pilot data demonstrated that minor differences in operative procedure or postoperative care translated into major differences in stroke outcome, the current study was undertaken to systematically identify important surgical, technical, and anatomic considerations required to obtain consistent results in a murine model of focal cerebral ischemia. When strokes are created in a rigidly controlled manner, differences, due to the absence (or overexpression) of a single gene product, should be readily discernable.

This study presents a detailed rendering of a reproducible murine model of focal cerebral infarction based on modifications of the original rat model (26). This study identifies procedural variables that have a large impact on stroke outcome which have not been previously reported in technical descriptions of murine stroke models. These variables include suture length and gauge, methods of vascular control, temperature regulation in mice, and differences between strains commonly used in the breeding of transgenic animals. As the model described lends itself to the

study of either permanent or transient focal cerebral ischemia, evidence is presented that with carefully chosen ischemia times, infarct volume and mortality in reperfused animals can be made to approximate those seen with permanent occlusion. Understanding potential model-dependent sources of variability in stroke outcome can help to clarify divergent results between different laboratories. Adoption of a standardized model which yields consistent results is an important first step towards the use of transgenic mice in the study of the pathophysiology of stroke.

Materials and methods:

Animal Purchase and Anesthesia: Male mice of three different strains (C57 BlackJ6, CD-1 and 129J) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were eight to ten weeks of age and weighed between 18-37 grams (as indicated) at the time of experiments. Mice were anesthetized with an intraperitoneal injection of 0.3 ml of ketamine (10 mg/cc) and xylazine (0.5 mg/cc). An additional dose of 0.1 cc was given prior to withdrawal of the catheter in animals undergoing transient ischemia. On the day following surgery, anesthesia was repeated immediately prior to laser doppler flow measurement and humane euthansia. These procedures have been approved by the Institutional Animal Care and Use Committee at Columbia University, and are in accordance with AALAC guidelines for the humane care and use of laboratory animals.

Surgical Set-up: The animal was positioned supine on a gauze pad which rests on a temperature controlled operating surface (Yellow Springs Instruments, Inc.[YSI], Yellow Springs, OH). A rectal temperature probe (YSI) was inserted, in order to regulate the temperature of the operating surface to maintain a constant animal core temperature of 36-38 °C. To facilitate exposure, the right hindpaw and left forepaw were taped to the operating surface, the right forepaw was taped to the animal's chest, and the tail was taped to the rectal probe (**Figure 12A**). A midline neck incision

was made by gently lifting the loose skin between the manubrium and the jaw and excising a 1 cm^2 circle of skin. The paired midline submandibular glands directly underlying this area were bluntly divided, with the left gland left *in situ*. The right gland was retracted cranially with an small straight Sugita aneurysm clip (Mizutto America, Inc., Beverly, MA) secured to the table by a 4.0 silk and tape. The sternocleidomastoid muscle was then identified, and a 4.0 silk ligature placed around its belly. This ligature was drawn inferolaterally, and taped to the table, to expose the omohyoid muscle covering the carotid sheath. The exposure is shown in **Figure 12B**.

Operative Approach: Once the carotid sheath was exposed, the mouse and the temperature control surface were placed under an operating microscope (16-25X zoom, Zeiss, Thornwood, NY), with a coaxial light source used to illuminate the field. Under magnification, the omohyoid muscle was carefully divided with pickups. The common carotid artery (CCA) was carefully freed from its sheath, taking care not to apply tension to the vagus nerve (which runs lateral to the CCA). Once freed, the CCA was isolated with a 4.0 silk, taped loosely to the operating table. Once proximal control of the CCA was obtained, the carotid bifurcation was placed in view. The occipital artery, which arises from the proximal external carotid artery and courses postero-laterally across the proximal internal carotid artery (ICA) to enter the digastric muscle, was isolated at its origin, and divided using a Malis bipolar microcoagulator (Codman-Schurtleff, Randolph, MA). This enabled better visualization of the ICA as it courses posteriorly and cephalad underneath the stylohyoid muscle towards the skull base. Just before the ICA enters the skull it gives off a pterygopalatine branch, which courses laterally and cranially. This branch was identified, isolated, and divided at its origin, during which time the CCA-ICA axis straightens. A 4.0 silk suture was then placed around the internal carotid artery for distal control, the end of which was loosely taped to the operating surface.

Next, the external carotid artery was placed in view. Its cranio-medial course was skeletonized and its first branch, the superior thyroid artery, was cauterized and divided. Skeletonization was subsequently carried out distally by elevation of the hyoid bone to expose the artery's bifurcation into the lingual and maxillary arteries. Just proximal to this bifurcation the external carotid was cauterized and divided. Sufficient tension was then applied to the silk sutures surrounding the proximal common, and distal internal, carotid arteries to occlude blood flow, with care taken not to traumatize the arterial wall. Tape on the occluding sutures was readjusted to maintain occlusion.

Introduction and Threading of the Occluding Intraluminal Suture: Immediately following carotid occlusion, an arteriotomy was fashioned in the distal external carotid wall just proximal to the cauterized area. Through this arteriotomy, a heat-blunted 5.0 or 6.0 nylon suture (as indicated in the Results section) was introduced (**Figures 12C and 12D**). As the suture was advanced to the level of the carotid bifurcation, the external stump was gently retracted caudally directing the tip of the suture into the proximal ICA. Once the occluding suture entered the ICA, tension on the proximal and distal control sutures was relaxed, and the occluding suture was slowly advanced up the ICA towards the skull base under direct visualization (beyond the level of the skull base, sight of the occluding suture is lost). Localization of the distal tip of the occluding suture across the origin of the middle cerebral artery (MCA) (proximal to the origin of the anterior cerebral artery) was determined by the length of suture chosen (12 mm or 13 mm as indicated in the Results section, shown in **Figure 12C**), by laser doppler flowmetry (see Ancillary physiological procedures section), and by post-sacrifice staining of the cerbral vasculature (see below). After placement of the occluding suture was complete, the external carotid artery stump was cauterized to prevent bleeding through the arteriotomy once arterial flow was reestablished.

Completion of Surgical Procedure: For all of the experiments shown, the duration of carotid occlusion was less than two minutes. To close the incision, the sutures surrounding the proximal and distal CCA, as well as the sternocleidomastoid muscle, were cut and withdrawn. The aneurysm clip was removed from the submandibular gland and the gland was laid over the operative field. The skin edges were then approximated with one surgical staple and the animal removed from the table.

Removal of the Occluding Suture to Establish Transient Cerebral Ischemia: Transient cerebral ischemia experiments required reexploration of the wound to remove the occluding suture. For these experiments, initial wound closure was performed with a temporary aneurysm clip rather than a surgical staple to provide quick access to the carotid. Proximal control with a 4-0 silk suture was reestablished prior to removal of the occluding suture to minimize bleeding from the external carotid stump. During removal of the occluding suture, cautery of the external carotid artery stump was begun early, before the distal suture has completely cleared the stump. Once the suture was completely removed, the stump is more extensively cauterized. Reestablishment of flow in the extracranial internal carotid artery was confirmed visually and the wound was closed as for permanent focal ischemia described above. Confirmation of intracranial reperfusion was accomplished with laser doppler flowmetry (see Ancillary physiological procedures section).

Calculation of Stroke Volume: Twenty-four hours after middle cerebral artery occlusion, surviving mice were reanesthetized with 0.3 cc of ketamine (10 mg/ml) and xylazine (0.5 mg/ml). After final weights, temperatures and cerebral blood flow readings were taken (as described below), animals were perfused with 5 ml of a 0.15 % solution of methylene blue and saline to enhance visualization of the cerebral arteries. Animals were then decapitated, and the brains were removed. Brains were then

inspected for evidence of correct catheter placement, as evidenced by negative staining of the vascular territory subtended by the MCA, and placed in a mouse brain matrix (Activational Systems Inc., Warren, MI) for 1 mm sectioning. Sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37 °C, and placed in 10% formalin (5). After TTC staining, infarcted brain was visualized as an area of unstained (white) tissue in a surrounding background of viable (brick red) tissue. Serial sections were photographed and projected on tracing paper at a uniform magnification; all serial sections were traced, cut out, and the paper weighed by a technician blinded to the experimental conditions. Under these conditions, infarct volumes are proportional to the summed weights of the papers circumscribing the infarcted region, and were expressed as a percentage of the right hemispheric volume. These methods have been validated in previous studies (3,12,15,16).

Ancillary Physiological Studies:

Ancillary physiogical studies were performed on each of the three different strains used in the current experiments, immediately prior to and after the operative procedure. Systemic blood pressures were obtained by catheterization of the infrarenal abdominal aorta, and measured using a Grass Model 7 polygraph (Grass Instrument Co., Quincy, MA). An arterial blood sample was obtained from this infrarenal aortic catheter; arterial pH, pCO_2 (mm Hg), pO_2 (mm Hg) and hemoglobin oxygen saturation (%) were measured using a Blood Gas Analyser and Hemoglobinometer (Grass Instrument Co., Quincy, MA). Because of the need for arterial puncture and abdominal manipulation to measure these physiologic parameters, animals were designated solely for these measurements (stroke volumes, neurologic outcome, and cerebral blood flows were not measured in these same animals).

Transcranial measurements of cerebral blood flow were made using

laser doppler flowmetry (Perimed, Inc., Piscataway, NJ) after reflection of the skin overlying the calvarium, as previously described (10) (transcranial readings were consistently the same as those made after craniectomy in pilot studies). To accomplish these measurements, animals were placed in a stereotactic head frame, after which they underwent midline skin incision from the nasion to the superior nuchal line. The skin was swept laterally, and a 0.7 mm straight laser doppler probe (model #PF2B) was lowered onto the cortical surface, wetted with a small amount of physiologic saline. Readings were obtained 2 mm posterior to the bregma, both 3 mm and 6 mm to each side of midline using a sterotactic micromanipulator, keeping the angle of the probe perpendicular to the cortical surface. Relative cerebral blood flow measurements were made immediately after anesthesia, after occlusion of the MCA, and immediately prior to euthanasia, and are expressed as the ratio of the doppler signal intensity of the ischemic compared with the nonischemic hemisphere. For animals subjected to transient cerebral ischemia, additional measurements were made just before and just after withdrawal of the suture, initiating reperfusion.

The surgical procedure/intraluminal MCA occlusion was considered to be technically adequate if ≥50% reduction in relative cerebral blood flow was observed immediately following placement of the intraluminal occluding catheter (15 of the 142 animals used in this study [10.6%] were exluded due to inadequate drop in blood flow at the time of occlusion). These exclusion criteria were shown in preliminary studies to yield levels of ischemia sufficient to render consistent infarct volumes by TTC staining. Reperfusion was considered to be technically adequate if cerebral blood flow at catheter withdrawal was at least twice occlusion cerebral blood flow (13/17 animals in this study [76%]).

Temperature: Core temperature during the peri-infarct period was carefully controlled throughout the experimental period. Prior to

surgery, a baseline rectal temperature was recorded (YSI Model 74 Thermistemp rectal probe, Yellow Springs Instruments, Inc., Yellow Springs, OH). Intraoperatively, temperature was controlled using a thermocouple-controlled operating surface. Following MCA occlusion, animals were placed for 90 minutes in an incubator, with animal temperature maintained at 37°C using the rectal probe connected via thermocouple to a heating source in the incubator. Temperature was similarly controlled in those animals subjected to transient ischemia, including a 45 minute (ischemic) period as well as a 90 minute post-ischemic period in the incubator. Following placement in the core-temperature incubator, animals were returned to their cages for the remaining duration of pre-sacrifice observation.

Neurological Exam: Prior to giving anesthesia at the time of euthanasia, mice were examined for obvious neurological deficit using a four-tiered grading system: (1) normal spontaneous movements, (2) animal circling towards the right, (3) animal spinning to the right, (4) animal crouched on all fours, unresponsive to noxious stimuli. This system was shown in preliminary studies to accurately predict infarct size, and is based on systems developed for use in rats (6).

Data Analysis: Stroke volumes, neurologic outcome scores, cerebral blood flows and arterial blood gas data were compared using an unpaired Student's t-test. Values are expressed as means ± SEM, with a $p < 0.05$ considered statistically significant. Mortality data, where presented was evaluated using chi-squared analysis.

Results:

Effects of Strain: Three different commonly used mouse strains (CD1, C57/Bl6, and 129J) were used to compare the variability in stroke outcome following permanent focal cerebral ischemia. To establish that there were no gross anatomic differences in collateralization of the cerebral circulation, the Circle of Willis

was visualized using India ink in all three strains **(Figure 13)**. These studies failed to reveal any gross anatomic differences. Mice of similar sizes (20 ± 0.8 g, 23 ± 0.4 g, and 23 ± 0.5 g for 129J, CD1, and C57Bl mice, respectively) were then subjected to permanent focal ischemia under normothermic conditions using a 12 mm length of 6-0 nylon occluding suture. Significant strain-related differences in infarct volume were noted, with infarcts in 129J mice being significantly smaller than those observed in CD1 and C57/Bl6 mice despite identical experimental conditions **(Figure 14A)**. Differences in infarct size were paralleled by neurological exam, with the highest scores (i.e., most severe neurologic damage) being seen in the C57/Bl6 and CD1 mice **(Figure 14B)**.

To determine the relationship between infarct volume and cerebral blood flow to the core region, laser doppler flowmetry was performed through the thin murine calvarium. No preoperative strain-related differences in cerebral blood flow were observed, corresponding to the lack of gross anatomic differences in vascular anatomy **(Figure 13)**. Measurement of cerebral blood flow immediately following insertion of the occluding catheter revealed that similar degees of flow reduction were created by the procedure (the percentage of ipsilateral/contralateral flow immediately following insertion of the obstructing catheter was 23 ± 2%, 19 ± 2%, 17 ± 3% for 129J, CD1, and C57/Bl6 mice, respectively). Not surprisingly, blood flow to the core region measured at 24 hours just prior to euthanasia demonstrated the lowest blood flows in those animals with the most severe neurologic injury **(Figure 14C)**.

Anatomic and Physiologic Characteristics of Mice: Baseline arterial blood pressures, as well as arterial blood pressures following middle cerebral artery occlusion, were nearly identical for all animals studied, and were not effected by mouse strain or size **(Table I)**. Analysis of arterial blood for pH, pCO_2, and hemoglobin oxygen saturation (%) similarly revealed no significant differences **(Table I)**.

Effect of Animal Size and Bore of the Occluding Suture: To investigate the effects of mouse size on stroke outcome, mice of two different sizes (23 ±0.4 g and 31 ±0.7 g) were subjected to permanent focal cerebral ischemia. To eliminate other potential sources of variability in these experiments, experiments were performed under normothermic conditions in mice of the same strain (CD1), using occluding sutures of identical length and bore (12 mm 6-0 nylon). Under these conditions, small mice (23 ± 0.4 g) sustained consistently large infarct volumes (28 ± 9% of ipsilateral hemisphere). Under identical experimental conditions, large mice (31 ± 0.7 g) demonstrated much smaller infarcts (3.2 ± 3%, p=0.02, **Figure 15A**), less morbidity on neurological exam **(Figure 15B)**, and a tendency to maintain higher ipsilateral cerebral blood flow following infarction than smaller animals **(Figure 15C)**.

Because it was hypothesized that the reduction in infarct size infarcts in these large animals was related to a mismatch in diameter/length between occluding suture and the cerebral blood vessels, longer/thicker occluding sutures were fashioned (13 mm, 5-0 nylon) for use in these larger mice. Large CD1 mice (34 ± 0.8 g) which underwent permanent occlusion with these larger occluding sutures sustained a marked increase in infarct volumes (50 ± 10% of ipsilateral hemisphere, p<0.0001 compared with large mice infarcted with the smaller occluding suture, **Figure 15A**). These larger mice infarcted with larger occluding sutures demonstrated higher neurologic deficit scores **(Figure 15B)** and lower ipsilateral cerebral blood flows **(Figure 15C)** compared with similarly large mice infarcted with smaller occluding sutures.

Effects of Temperature: To establish the role of perioperative hypothermia on the stroke volumes and neurologic outcomes following MCA occlusion, small C57/Bl6 mice (22 ± 0.4g) were subjected to permanent MCA occlusion with 12 mm 6-0 gauge suture, with

normothermia maintained for two different durations; Group 1 ("Normothermia") was operated as described above, maintaining temperature at 37 °C from the preoperative period until 90 minutes post-occlusion. Group 2 animals ("Hypothermia") were maintained at 37 °C from preop to only 10 minutes post-occlusion, as has been described previously (14). Within 45 minutes following removal from the thermocouple-controlled warming incubator, core temperature in this second group of animals dropped to 33.1 ± 0.4 °C (and dropped further to 31.3 ± 0.2 °C at 90 minutes). Animals operated under conditions of prolonged normothermia (Group 1) exhibited larger infarct volumes (32 ± 9%) than hypothermic (Group 2) animals (9.2 ± 5%, p = 0.03, **Figure 16A**). Differences in infarct volume were mirrored by differences in neurological deficit (3.2 ±0.4 vs. 2.0 ±0.8, p=0.02, **Figure 16B**), but were largely independent of cerebral blood flow (52 ± 5 vs. 52 ± 7, p = NS, **Figure 16C**).

Effects of Transient MCA Occlusion: Because reperfusion injury has been implicated as an important cause of neuronal damage following cerebrovascular occlusion (25), a subset of animals was subjected to a transient (45 minute) period of ischemia followed by reperfusion as described above, and comparisons made with those animals which underwent permanent MCA occlusion. The time of occlusion was chosen on the basis of preliminary studies (not shown) which demonstrated unacceptibly high mortality rates (>85%) with 180 minutes of ischemia and rare infarction (<15%) with 15 minutes of ischemia. To minimize the confounding influence of other variables, other experimental conditions were kept constant (small (22.5 ± 0.3 g) C57/Bl6 mice were used, the occluding suture consisted of 12 mm 6-0 nyon, and experiments were performed under normothermic conditions). The initial decline in CBF immediately post-occlusion were similar in both groups (16 ± 2% vs 17 ± 3%, for transient vs permanent occlusion groups, respectively, p=NS). Reperfusion was confirmed both by laser doppler (2.3-fold increase in blood flow following removal of the occluding suture to 66 ±

13%), and visually by intracardiac methylene blue dye injection in representative animals. Infarct sizes (29 ± 10% vs. 32 ± 9%), neurologic deficit scores (2.5 ±0.5 vs. 3.2 ±0.4), and sacrifice cerebral blood flow (46 ±18% vs. 53 ±5%) were quite similar between between animals subjected to transient cerebral ischemia and reperfusion and those subjected to permanent focal cerebral ischemia (p =NS, for all groups) **(Figures 17A-17C)**.

Discussion:

The growing availability of genetically altered mice has led to an increasing use of murine models of focal cerebral ischemia to impute specific gene products in the pathogenesis of stroke. Although recent publications describe the use of an intraluminal suture to occlude the middle cerebral artery to create permanent and/or transient cerebral ischemia in mice, there has been only scant description of the necessary modifications of the original technical report in rats (8,14,17-19,24,26). The experiments described herein not only provide a detailed technical explanation of a murine model suitable for either permanent or transient focal middle cerebral artery ischemia, but also address potential sources of variability in the model.

Importance of Strain:

One of the most important potential sources of variability in the murine cerebral ischemia model described herein is related to the strain of animal used. The data suggest that, of the three strains tested, 129J mice are particularly resistant to neurologic injury following MCA occlusion. Although Barone similarly found differences in stroke volumes between 3 strains of mice (BDF, CFW and BALB/C), these differences were ascribed to variations in the posterior communicating arteries in these strains (4). As anatomical differences in cerebrovascular anatomy were not grossly apparent in the study (**Figure 13**), the data suggests that non-anatomic strain-related differences are also important in outcome following MCA occlusion.

As stroke outcome differs significantly between 2 strains of mice (129J and C57/Bl6) commonly used to produce transgenic mice via homologous recombination in embryonic stem cells (11), the data suggest an important caveat to experiments performed with transgenic mice. Because early founder progeny from the creation of transgenic animals with these strains have a mixed 129J / C57/Bl6 background, ideally experiments should be performed either with sibling controls or after a sufficient number of backcrossings to ensure strain purity.

Importance of Size:

Larger animals require a longer and thicker intralumenal suture to sustain infarction volumes which are consistent with those obtained in smaller animals with smaller occluding sutures. Size matching of animal and suture appear to be important not only to produce consistent cerebral infarction, but whereas too small a suture leads to insufficient ischemia, too large a suture leads to frequent intracerebral hemorrhage and vascular trauma (unpublished observation).

The use of animals of similar size is important not only to minimize potential age-related variability in neuronal susceptability to ischemic insult, but also to ensure that small differences in animal size do not obfuscate meaningful data comparison. In this example, it is demonstrated that size differences of as little as 9 grams can have a major impact on infarct volume and neurologic outcome following cerebral ischemia. Further experiments using larger bore occluding suture in larger animals suggest that the increased propensity of smaller animals to have larger strokes was not due to a relative resistance of larger animals to ischemic neuronal damage, but was rather due to small size of the suture used to occlude the MCA in large animals. Although these data were obtained using CD1 mice, similar studies have been performed and found these results to be true with other mouse strains as well, such as C57/Bl6 (unpublished data).

Previously published reports use mice of many different sizes (from 21 g to 35 g), as well as different suture diameters and lengths which are often unreported (14,17). The studies indicate that animal and suture size are important methodological issues which must be addressed in scientific reports.

Importance of Temperature:

It has long been recognized that hypothermia protects a number of organs from ischemic injury, including the brain. Studies performed in rats have demonstrated that intraischemic hypothermia up to 1 hour post-MCA occlusion is protective (2,15), reducing both mortality and infarct volumes with temperatures of 34.5 degrees. Although these results have been extrapolated to murine models of cerebral ischemia in that studies often describe maintenance of normothermia in animals, the post-MCA occlusion temperature monitoring periods have been extremely brief ("immediately after surgery" or "10 minutes after surgery") (4,14). The results indicate that animals fail to autoregulate their temperature beyond these brief durations, becoming severely hypothermic during the postoperative period, and that temperature differences up to 90 minutes following MCA occlusion can have a profound effect on indices of stroke outcome following MCA occlusion (longer durations of normothermia were not studied). While others have ensured normothermia using a feedback system based on rectal temperature similar to the one described herein, the duration of normothermia is often not specified (17). The results argue for clear identification of methods for monitoring and maintaining temperature, as well as the durations involved, so that experimental results can be compared both within and between Centers studying the pathophysiology of stroke.

Transient vs Permanent Occlusion:

The pathophysiology of certain aspects of permanent cerebral ischemia may well be different from that of cerebral ischemia followed by reperfusion, so it was important that a model be

described which permitted analysis of either condition. Although differences between these two models were not extensively tested in the current series of experiments, under the conditions tested (45 minutes of ischemia followed by 23 hours of reperfusion), no significant differences were found in any index of stroke outcome. Variable durations of ischemia and reperfusion have been reported in other murine models of transient cerebral ischemia, with ischemic times ranging from 10 minutes to 3 hours and reperfusion times ranging from 3 to 24 hours (17,24). Studies in rats have shown that short periods of ischemia followed by reperfusion are associated with smaller infarcts than permanent occlusion (21,25). However as the duration of ischemia increases beyond a critical threshold (between 120 and 180 minutes), reperfusion is associated with larger infarcts (7,21,26). For the current series of experiments, the durations of ischemia and reperfusion were chosen so as to obtain infarcts comparable to those observed following permanent MCA occlusion, which is likely to explain why the data failed to show differences between permanent and transient ischemia. These durations in the transient model were chosen after pilot experiments revealed that shorter ischemic durations (15 minutes) rarely led to infarction, whereas 180 minutes of occlusion followed by reperfusion led to massive infarction and nearly 100% mortality within 4-6 hours in normothermic animals (unpublished observation). Although indices of stroke outcome may be measured earlier than 24 hours, the 24 hour observation time was elected because observation at this time permits the study of delayed penumbral death, which is likely to be clinically relevant to the pathophysiology of stroke in humans. Furthermore, 24 hours has been shown in a rat model to be sufficient for full infarct maturation (3,12,15,16).

Technical Aspects of the Murine Model:

Technical aspects of the surgery needed to create focal cerebral ischemia in mice differ in certain important respects from that in rats. Self-retaining retractors, which have been advocated in

previous reports in rats (26), are unweildy in mice. Suture-based retraction secured with tape provides a superior alternative. In rats, clip occlusion of the proximal and distal carotid artery after mobilization of the external carotid artery has been reported (26), but creates more carotid trauma and hemmorhage in mice. Without distal internal carotid control, which has not been previously described in mice, backbleeding from the external carotid artery is consistently uncontrollable. Using the techniques described in this paper, surgery can be completed with virtually no blood loss, which is especially important given the small blood volume in mice.

Unlike the rat model, the occlusion and transection of the external carotid artery branches and the pterygopalatine artery in the murine model is achieved with electrocautery alone. Previous reports of murine surgery have been unclear as to whether or not the pterygopalatine artery was taken (17,24). Others have described a method with permanent occlusion of the common carotid artery and trans-carotid insertion of the suture without attention to either the external carotid system or the pterygopalatine artery. While effective for permanent occlusion, this latter method makes reperfusion studies impossible.

The method of reperfusion originally described in the rat requires blind catheter withdrawal without anesthesia (26). When attempted in pilot studies in mice, several animals hemorrhaged. Therefore, a method of suture removal under direct visualization in the anesthetized animal was developed, which not only allows visual confirmation of extracranial carotid artery reperfusion, but also affords meticulous hemostasis. Further, the method permits immediate pre- and post-reperfusion laser doppler flowmetry readings in the anesthetized animal.

These laser doppler flowmetry readings are similar to those described by Kamii et al. and Yang et al. in that the readings are

made intermittantly and with the use of a stereotactic micromanipulator (17,24). The readings differ, however, in that the coordinates used (2 mm posterior and 3 and 6 mm lateral to the bregma) are slightly more lateral and posterior than the previously published core and penumbral coordinates (1 mm posterior and 2 mm and 4.5 mm lateral to the bregma). These coordinates, which were adopted based on pilot studies, are the same as those used by Huang et al (14).

Conclusion:

These studies demonstrate specific technical aspects of a murine model of focal cerebral ischemia and reperfusion which permits reproducibility of measurements between different laboratories. In addition, these studies provide a framework for understanding important procedural variables which can greatly impact on stroke outcome, which should lead to a clear understanding of non-procedure related differences under investigation. Most importantly, this study points to the need for careful control of mouse strain, animal and suture size, and temperature in experimental as well as control animals. Conditions can be established so that stroke outcome is similar between models of permanent focal cerebral ischemia and transient focal cerebral ischemia, which should facilitate direct comparison and permit the study of reperfusion injury. The model described in this study should provide a cohesive framework for evaluating the results of future studies in transgenic animals, to facilitate an understanding of the contribution of specific gene products in the pathophysiology of stroke.

Table I. Pre- and post-operative physiologic parameters. MAP, mean arterial pressure; pCO_2, partial pressure of arterial CO_2 (mm Hg); O_2 Sat, O_2 saturation (%); Hb, hemoglobin concentration (g/dl); Preoperative, anesthetized animals prior to carotid dissection; Sham, anesthetized animals undergoing the surgical described in the text, immediately prior to introduction of the occluding suture;

Stroke, anesthetized animals undergoing the surgical described in the text, immediately after introduction of the occluding suture. p=NS for all between-group comparisons. (data shown is for small 22 gram C57/Bl6 mice).

PARAMETER	PREOPERATIVE	SHAM	STROKE
MAP	102 ± 5.5	94 ± 1.9	88 ± 4.9
pH	7.27 ± 0.02	7.23 ± 0.04	7.28 ± 0.01
pCO_2	46 ± 1.3	44 ± 1.3	47 ± 3.5
O_2 Sat	89 ± 1.6	91 ± 1.8	85 ± 2.2
Hb	14.6 ± 0.42	14.3 ± .12	14.2 ± 0.12

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EXAMPLE 4: Exacerbation of Cerebral Injury In Mice Which Express the P-Selectin Gene: Identication of P-selectin Blockade as a New Target for the Treatment of Stroke

There is currently a stark therapeutic void for the treatment of evolving stroke. Although P-selectin is rapidly expressed by hypoxic endothelial cells *in vitro*, the functional significance of P-selectin expression in stroke remains unexplored. In order to identify the pathophysiological consequences of P-selectin expression and to identify P-selectin blockade as a potential new approach for the treatment of stroke, experiments were performed using a murine model of focal cerebral ischemia and reperfusion. Early P-selectin expression in the post-ischemic cerebral cortex was demonstrated by the specific accumulation of radiolabelled anti-murine P-selectin IgG. In parallel experiments, neutrophil accumulation in the ischemic cortex of mice expressing the P-selectin gene (PS +/+) was significantly greater than that demonstrated in homozygous P-selectin null mice (PS -/-). Reduced neutrophil influx was accompanied by greater postischemic cerebral reflow (measured by laser doppler) in the PS -/- mice. In addition, PS -/- mice demonstrated smaller infarct volumes (five-fold reduction, $p < 0.05$) and improved survival compared with PS +/+ mice (88% vs. 44%, $p < 0.05$). Functional blockade of P-selectin in PS +/+ mice using a monoclonal antibody directed against murine P-selectin also improved early reflow and stroke outcome compared with controls. These data are the first to

demonstrate a pathophysiological role for P-selectin in stroke, and suggest that P-selectin blockade may represent a new therapeutic target for the treatment of stroke.

INTRODUCTION

Ischemic stroke consitutes the third leading cause of death in the United States today [1]. Until very recently, there has been no direct treatment to reduce cerebral tissue damage in evolving stroke. Although the NINDS [2] and ECASS[3] rt-PA acute stroke studies have suggested that there are potential therapeutic benefits of early reperfusion [4], the increased mortality observed following streptokinase treatment of acute ischemic stroke [5] highlights the sobering fact that there is at the present time no clearly effective treatment for evolving stroke. This void in the current medical armamentarium for the treatment of stroke has led to a number of innovative approaches [6], yet other than rt-PA, none have reached the clinical realm. To identify a potential safe and efficacious treatment for evolving stroke, attention has been focussed on the deleterious role of recruited neutrophils. Recent work in a murine model of reperfused stroke has demonstrated that depletion of neutrophils (PMNs) prior to stroke minimizes cerebral tissue injury and improves functional outcome [7]; mice which lack the specific cell adhesion molecule, ICAM-1, are similarly protected [7]. P-selectin, a molecule which can be rapidly translocated to the hypoxic endothelial surface from pre-formed storage sites [8], is an important early mediator of the neutrophil rolling [9], which facilitates ICAM-1-mediated neutrophil arrest. Although P-selectin is expressed in primate stroke [10], there are no published data which addresses the functional significance of P-selectin expression in any model of either reperfused or nonreperfused stroke.

To explore the pathophysiological role of P-selectin in stroke, a murine model of focal cerebral ischemia and reperfusion [11] was employed using both wild type mice and mice which were homozygous null for the P-selectin gene [9] and a strategy of administering a functionally blocking P-selectin antibody. This study confirms not only that P-selectin expression following middle cerebral artery occlusion is associated with reduced cerebral reflow following reperfusion and a worse outcome following stroke, but that P-selectin blockade confers a significant degree of postischemic cerebral protection. These studies represent the first demonstration of the pathophysiological role of P-selectin expression in stroke, and suggest the exciting possibility that anti-P-selectin strategies may prove useful for the treatment of reperfused stroke.

METHODS

Mice: Experiments were performed with transgenic P-selectin deficient mice, created as previously reported [9] by gene targeting in J1 embryonic stem cells, injected into C57BL/6 blastocysts to obtain germline transmission, and backcrossed to obtain homozygous null P-selectin mice (PS -/-). Experiments were performed with PS -/- or wild-type (PS +/+) cousin mice from the third generation of backcrossings with C57BL/6J mice. Animals were seven to twelve weeks of age and weighed between 25-36 grams at the time of experiments.

Transient Middle Cerebral Artery Occlusion: Mice were anesthetized (0.3 cc of 10 mg/cc ketamine and 0.5 mg/cc xylazine, i.p.), and positioned supine on a rectal temperature-controlled operating surface (Yellow Springs Instruments, Inc., Yellow Springs, OH). Animal core temperature was maintained at 37 ± 1°C intraoperatively and for 90 minutes post-operatively. A midline neck incision was created to expose the right carotid sheath under the operating microscope (16-25X zoom, Zeiss, Thornwood, NY). The common carotid artery was isolated with a 4-0 silk, and the occipital,

pterygopalatine, and external carotid arteries were each isolated and divided. Middle cerebral artery occlusion (MCAO) was accomplished by advancing a 13 mm heat-blunted 5-0 nylon suture via the external carotid stump. After placement of the occluding suture, the external carotid artery stump was cauterized, and the wound was closed. After 45 minutes, the occluding suture was withdrawn to establish reperfusion. These procedures have been previously described in detail [9].

Measurement of cerebral cortical blood flow: Transcranial measurements of cerebral blood flow were made using laser doppler (Perimed, Inc., Piscataway, NJ), as previously described [12]. Using a 0.7 mm straight laser doppler probe (model #PF303, Perimed, Piscataway, NJ) and previously published landmarks (2 mm posterior to the bregma, 6 mm to each side of midline) [11,13], relative cerebral blood flow measurements were made as indicated; immediately after anesthesia, 1 and 10 minutes after occlusion of the middle cerebral artery, as well as after 30 minutes, 300 minutes and 22 hours of reperfusion. Data are expressed as the ratio of the doppler signal intensity of the ischemic compared with the nonischemic hemisphere. Although this method does not quantify cerebral blood flow per gram of tissue, use of laser doppler flow measurements at precisely defined anatomic landmarks serves as a means of comparing cerebral blood flows in the same animal serially over time. The surgical procedure was considered to be technically adequate if ≥ 50% reduction in relative cerebral blood flow was observed immediately following placement of the intraluminal occluding suture. These methods have been used in previous studies [7,11].

Cerebrovascular anatomy was determined in representative animals in the following manner. Mice were anesthetized, and an antemortem injection (0.1 mL) of India ink:carbon black:methanol:physiological saline (1:1:1:1, v:v:v:v) was given by left ventricular puncture. Brains were prepared by rapid decapitation followed by immersion in 10% formalin at 4°C for 2 days, after which the inferior surfaces

were photographed to demonstrate the vascular pattern of the Circle of Willis.

Preparation and administration of ^{125}I-labelled proteins and ^{111}In-labelled murine neutrophils: Radioiodinated antibodies were prepared as follows. Monoclonal rat anti-murine P-selectin IgG (Clone RB 40.34, Pharmingen Co., San Diego, CA) [14] and non-immune rat IgG (Sigma Chemical Co., St. Louis, MO) were radiolabeled with ^{125}I by the lactoperoxidase method [15] using Enzymobeads (Bio-Rad, Hercules, CA). Radiolabelled PMNs were prepared in the following manner. Citrated blood from wild type mice was diluted 1:1 with NaCl (0.9%) followed by gradient ultracentrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Following hypotonic lysis of residual erythrocytes (20 sec exposure to distilled H_2O followed by reconstitution with 1.8% NaCl), the PMNs were suspended in phosphate buffered saline (PBS). Neutrophils (5-7.5 x 10^6) were suspended in PBS with 100 μCi of ^{111}Indium oxine (Amersham Mediphysics, Port Washington, NY), and subjected to gentle agitation for 15 minutes at 37 °C. After washing with PBS, the PMNs were gently pelleted (450 x g), and resuspended in PBS to a final concentration of 1.0 x 10^6 cells/mL.

Neurological Exam: Prior to giving anesthesia mice were examined for neurological deficit 22 h after reperfusion using a four-tiered grading system [11]: a score of [1] was given if the animal demonstrated normal spontaneous movements; a score of [2] was given if the animal was noted to be turning towards the ipsilateral side; a score of [3] was given if the animal was observed to spin longitudinally (clockwise when viewed from the tail); a score of [4] was given if the animal was unresponsive to noxious stimuli. This scoring system has been previously described in mice [7,11], and is based upon similar scoring systems used in rats [16,17].

Calculation of Infarct Volumes: After neurologic examination, mice were anesthesized and final cerebral blood flow

measurements obtained. Humane euthanasia was performed by decapitation, and brains were removed and placed in a mouse brain matrix (Activational Systems Inc., Warren, MI) for 1 mm sectioning. Sections were immersed in 2% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC, Sigma Chemical Co., St. Louis, MO) in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37°C, and placed in 10% formalin [18]. Infarcted brain was visualized as an area of unstained tissue. Infarct volumes were calculated from planimetered serial sections and expressed as the percentage of infarct in the ipsilateral hemisphere. This method of calculating infarct volumes has been used previously [7,11,13,18], and has been correlated with the other functional indices of stroke outcome which are described above.

Administration of Unlabeled Antibodies, Radiolabelled PMNs, and Radiolabeled Antibodies: For experiments in which unlabeled antibodies were administered, one of two different antibody types was used; either a blocking monoclonal rat anti-murine P-selectin IgG (Clone RB 40.34, Pharmingen Co., San Diego, CA) [14,19,20] or non-immune rat IgG (Sigma Chemical Co., St. Louis, MO). Antibodies were prepared as 30 μg in 0.2 mL phosphate buffered saline containing 0.1% bovine serum albumin, which was then administered into the penile vein 10 minutes prior to middle cerebral artery occlusion. In separate experiments, radiolabeled antibodies (0.15 mL, $\approx 2.6 \times 10^5$ cpm/μL) were injected intravenously 10 minutes prior to middle cerebral artery occlusion. In a third set of experiments, radiolabelled PMNs were administered intravenously 10 minutes prior to middle cerebral artery occlusion as a 100 μL injection (radiolabelled PMNs were admixed with physiologic saline to a total volume of 0.15 mL; $\approx 3 \times 10^6$ cpm/μL). For experiments in which unlabeled antibodies were administered, the time at which measurements were made are indicated in the text, using the methods described above to determine cerebral blood flow, infarction volumes, and mortality. For those experiments in which either radiolabelled antibodies or radiolabelled nPMNs were administered,

mice were sacrificed at the indicated time points and brains were immediately removed and divided into ipsilateral (postischemic) and contralateral hemispheres. Deposition of radiolabeled antibodies or neutrophils was measured and expressed as ipsilateral/ contralateral cpm.

Data Analysis: Cerebral blood flow, infarct volume, and ^{111}InPMN deposition were compared using Student's t-test for unpaired variables. Neurological deficit scores were compared using the Mann-Whitney U-test. Two way ANOVA was performed to test for significant differences between baseline and final (30 min) antibody deposition between the two groups (experimental vs sham). Student's t-test for unpaired variables was performed to evaluate within-group differences (baseline vs the 30 min. time point). Survival differences between groups was tested using contingency analysis with the Chi-square statistic. Values are expressed as mean ± SEM, with a p value < 0.05 considered statistically significant.

RESULTS

P-selectin Expression in Murine Stroke: Because P-selectin mediates the initial phase of leukocyte adhesion to activated endothelial cells [21], early cerebral P-selectin expression was examined in a murine model of reperfused stroke. Mice given a ^{125}I-labelled rat monoclonal anti-murine P-selectin IgG prior to surgery demonstrated a 216% increase in accumulation of the antibody at 30 minutes of reperfusion compared with sham operated animals ($p < 0.001$, **Figure 18A**). To demonstrate that this degree of antibody deposition in the reperfused hemisphere was due to P-selectin expression rather than nonspecific accumulation, comparison was made with identically-treated animals given a ^{125}I-labelled rat nonimmune IgG. These experiments demonstrated that there was significantly greater accumulation of the anti-P-selectin IgG than the nonimmune IgG ($p < 0.025$, **Figure 18A**), suggesting that P-selectin is expressed in the brain within 30 minutes of

reperfusion.

Neutrophil Accumulation in Murine Stroke: To delineate the time course over which PMN influx occurs following stroke, ^{111}In-labeled PMN accumulation was measured in wild type (PS +/+) mice prior to MCAO, immediately following and 10 minutes after MCAO, and at 30 min, 300 min, and 22 hrs of reperfusion. In PS +/+ mice, accumulation of PMNs begins early following the initiation of focal ischemia, and continues throughout the period of reperfusion **(Figure 18B)**. To establish the role for P-selectin in this postischemic neutrophil accumulation, experiments were performed using mice which were homozygous null for the P-selectin gene (PS -/-). PS -/- mice showed significantly reduced PMN accumulation following middle cerebral artery occlusion and reperfusion (**Figure 18B**).

Role of PS in Cerebrovascular No-reflow: To determine whether the reduction in PMN accumulation in PS -/- mice resulted in improved cerebral blood flow following the reestablishment of flow, serial measurements of relative CBF were obtained by laser doppler in both PS +/+ and PS -/- mice. Prior to the initiation of ischemia (**Figure 19, point a**), relative cerebral blood flows were nearly identical between groups. Middle cerebral artery occlusion (**Figure 19, point b**) was associated with a nearly identical drop in cerebral blood flow in both groups. Immediately prior to withdrawal of the intraluminal occluding suture at 45 minutes of ischemia (**Figure 19, point c**), cerebral blood flows had risen slightly, although they remained significantly depressed compared with baseline flows. Immediately following withdrawal of the occluding suture to initiate reperfusion (**Figure 19, point d**), cerebral blood flows in both groups increased to a comparable degree (≈60% of baseline in the PS -/- and PS +/+ mice). The immediate failure of the post-reperfusion cerebral blood flows to reach pre-occlusion levels is characteristic of cerebrovascular no-reflow [22], with the subsequent decline in post-reperfusion cerebral

blood flows representing delayed post-ischemic cerebral hypoperfusion [23]. By 30 minutes of reperfusion (**Figure 19, point e**), the cerebral blood flows between the two groups of animals had diverged, with PS -/- animals demonstrating significantly greater relative cerebral blood flows than the PS +/+ controls ($p < 0.05$). (**Figure 19, point f**). This divergence reflected significant differences in delayed post-ischemic cerebral hypoperfusion, and persisted for the 22 hour observation period.

Because variations in cerebrovascular anatomy have been reported to result in differences in susceptibility to experimental stroke in mice [24], India ink/carbon black staining was performed to visualize the the vascular pattern of the Circle of Willis in both in both PS -/- and PS +/+ mice. These experiments demonstrated that there were no gross anatomic differences in the vascular pattern of the cerebral circulation (**Figure 20**).

Stroke Outcome: The functional significance of P-selectin expression was tested by comparing indices of stroke outcome in PS -/- mice to those in PS +/+ controls. PS -/- mice were significantly protected from the effects of focal cerebral ischemia and reperfusion, based on a 77% reduction in infarct volume ($p < 0.01$) compared with P-selectin +/+ controls (**Figure 21A**). This reduction in infarct volume was accompanied by a trend towards reduced neurologic deficit ($p = 0.06$, **Figure 21B**) and increased survival ($p < 0.05$; **Figure 21C**) in the PS -/- animals.

Effect of P-selectin Blockade: After having observed the functional role of P-selectin expression in stroke using deletionally mutant mice, experiments were performed to determine whether pharmacological blockade of P-selectin could improve stroke outcome in PS +/+ mice. Using a strategy of administering a monoclonal rat anti-mouse P-selectin blocking antibody (clone RB 40.34, [14,19,20]) or nonimmune control rat IgG immediately prior to surgery, mice receiving the blocking antibody were observed to have

improved post-reperfusion cerebral blood flows by thirty minutes (**Figure 22A**), as well as reduced neurological deficits (**Figure 22B**), reduced cerebral infarction volumes (**Figure 22C**), and a trend towards reduced mortality compared with controls (**Figure 22D**).

DISCUSSION

Despite substantial progress in recent years in the primary prevention of stroke [1], therapeutic options to treat evolving stroke remain extremely limited [6]. Although the publication of two landmark trials last fall demonstrating reduced morbidity following treatment of ischemic stroke with rt-PA [2,3] was thought to usher in a new era of thrombolytic therapy in the treatment of stroke [4], enthusiasm has been tempered somewhat by the hemorrhagic transformation and increased mortality noted in patients with ischemic stroke treated with streptokinase [5]. These divergent trials make it more critical than ever that new safe therapies be developed to treat evolving stroke. Although restoration of blood flow to postischemic brain affords new opportunities for early therapeutic intervention, reperfusion is a double-edged sword. Given the cytotoxic potential of neutrophils [25], it is not surprising that neutrophil influx into postischemic brain tissue can lead to further damage and worsen outcome following experimental stroke [7,26-29]. Using a murine model of focal cerebral ischemia and reperfusion, an important contributory role for the cell adhesion molecule ICAM-1 in neutrophil accumulation at 22 hours following stroke was recently identified [7]. However, augmented cerebrovascular endothelial ICAM-1 expression requires *de novo* transcriptional and translational events, which requires time. In contrast, P-selectin, a membrane-spanning glycoprotein which mediates the earliest phases of neutrophil adhesion, may be mobilized from preformed storage pools to be rapidly expressed at the ischemic endothelial cell surface [8,30]. As the clinical trials of thrombolytic therapy for stroke demonstrate a narrow time window for potential benefit (within the first several hours of stroke

onset) [2,3,5], this suggests that strategies designed to interfere with the earliest phases of PMN adhesion might be of theoretical benefit in human stroke. These trials should result in greater numbers of patients presenting for earlier therepeutic intervention, increasing the need to address the issue of reperfusion injury in medically revascularized territories. In addition, these trials underscore the pressing need to understand the contributions of individual adhesion molecules to the pathogenesis of stroke.

Given the considerable body of literature describing the role of P-selectin in other models of ischemia and reperfusion [8,31-34], surprisingly little is known about the role of P-selectin in stroke. Knowledge of the specific role of P-selectin in the cerebral vasculature is important because adhesion molecule requirements vary between vascular beds and conditions under study. For instance, in a model of intestinal transplantation [35], anti-P-selectin antibodies did not reduce reperfusion injury, whereas anti-CD11/CD18 antibodies did. Although P-selectin blockade was ineffective at reducing PMN adhesion and albumin leakage in a rat mesentaric ischemia and reperfusion model,. ICAM-1 blockade was effective [36]. In a rat hindlimb ischemia/reperfusion model, the selectin requirements for PMN adhesion differed between the pulmonary and crural muscle vascular beds [33].

The only published study dealing with P-selectin in the ischemic brain is a histopathological description of primate stroke, in which P-selectin expression was increased in the lenticulostriate microvasculature [10]. Furthermore, there is no data which addresses the functional significance of this P-selectin expression. The current studies were undertaken to study whether P-selectin expression contributes to post-ischemic cerebral neutrophil accumulation, no-reflow, and tissue injury in a murine model of reperfused stroke. Using a recently established model of focal cerebral ischemia and reperfusion in mice [11], P-selectin expression

was demonstrated by increased deposition of radiolabelled antibody into the ischemic territory. In this technique, antibody deposition into the ischemic hemisphere was normalized to that in the nonischemic hemisphere in each animal, not only to minimize potential variations in injection volume or volume of distribution, but to enable comparison between animals given different antibodies. Because disruption of the endothelial barrier function in the ischemic cortex may augment nonselective antibody deposition, similar experiments were performed with a control rat IgG. These data show that the antibody which binds to P-selectin is deposited at an accelerated rate compared with the control antibody, suggesting that local P-selectin expression is augmented in the reperfused tissue. This data in the murine model parallels that reported in a baboon model of stroke [10], in which P-selectin expression was increased within 1 hour following the ischemic event.

The role of P-selectin expression in recruiting PMNs to the post-ischemic zone was demonstrated using a strategy in which accumulation of ^{111}In-labelled PMNs was measured. Although it was previously reported that by 22 hours, PMN accumulation is elevated in the ischemic hemisphere [7], the current time-course data demonstrate that PMN accumulation begins shortly after the onset of ischemia. Failure to express the P-selectin gene was associated with reduced PMN accumulation, suggesting the participation of P-selectin in post-ischemic cerebral PMN recruitment. However, the P-selectin null animals did demonstrate a modest (albeit less than control) neutrophil accumulation by 22 hours. This data indicates that P-selectin is not the exclusive effector mechanism responsible for postischemic cerebral PMN recruitment, and is consistent with the previous data that ICAM-1 also participates in post-ischemic PMN adhesion [7]. Furthermore, this data is not unlike that in which intra-abdominal instillation of thioglycollate in P-selectin deficient mice caused delayed (but not absent) PMN recruitment [9].

Because of the critical need to identify reasons for failed reperfusion, the current studies examined the role of P-selectin in delayed postischemic cerebral hypoperfusion [22,23], the phenomenon wherein blood flow declines during reperfusion, despite restoration of adequate perfusion pressures. In cardiac models of ischemia, no-reflow worsens as time elapses after reperfusion [37], suggesting an important role for recruited effector mechanisms, such as progressive microcirculatory thrombosis, vasomotor dysfunction, and PMN recruitment. Both P-selectin and ICAM-1-dependent adherence reactions [38] and PMN capillary plugging [39] have been shown in other models to participate in post-ischemic no-reflow. In the brain, PMNs have been implicated in post-ischemic cerebral no reflow [40,41], but the role of P-selectin in this process has not been elucidated. The current study uses a relatively noninvasive technique (laser doppler) to obtain serial measurements of relative cerebral blood flow, in order to establish the existence, time course, and P-selectin-dependence of post-ischemic cerebrovascular no-reflow. In these experiments, P-selectin null and control animals were subjected to virtually identical degrees of ischemia, and instantaneous recovery of blood flow following removal of the intraluminal occluding suture was the same in the two groups. However, cerebral blood flow declined in the time period following reperfusion in P-selectin +/+ animals. In sharp contrast, the PS -/- animals demonstrated only slight delayed post-ischemic cerebral hypoperfusion. This late (albeit limited) decline in cerebral blood flow by 22 hours is consistent with the modest PMN recruitment observed in the PS -/- animals over the same period. This again suggests that other effector mechanisms (such as ICAM-1) may be responsible for the late decline in cerebral blood flow in PS -/- animals.

The functional effects of P-selectin expression are clear from the current set of studies: animals which fail to express the P-selectin gene (or PS +/+ animals treated with a functionally blocking anti-P-selectin antibody) exhibit smaller infarcts,

improved survival, and survivors demonstrate improved neurologic outcomes compared with controls. When these data are considered along with previously published data demonstrating a deleterious role for ICAM-1 expression in stroke [7], it becomes increasingly apparent that there are multiple means for recruiting PMNs to post-ischemic cerbral cortex, and that blockade of each represents a potential strategy to improve stroke outcome in humans. Given the current recognition of the importance of timely reperfusion in halting the advancing wavefront of neuronal death following stroke, interfering with PMN adhesion at its earliest stages appears to be an attractive option for reducing morbidity and mortality. In fact, anti-adhesion molecule strategies may not only be beneficial in their own right (i.e., including patients ineligible for thrombolysis), but may extend the window of opportunity for thrombolytic intervention [42]. The current set of studies contributes to the understanding of pathophysiological mechanisms operative in reperfused stroke. These studies suggest the need for clinical trials of therapies for evolving stroke which optimize the reperfusion milieu to reduce PMN accumulation.

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EXAMPLE 5: P-Selectin Homozygous Null Mice Are Resistant to Focal Cerebral Ischemia and Reperfusion Injury

The role of neutrophils (PMNs) in potentiating focal ischemia reperfusion injury in the central nervous system remains controversial. An important early step in the capture of circulating PMNs by the vasculature is mediated by P-selectin expressed on postischemic endothelium. Although early and

persistent endothelial P-selectin expression has been described in brain microvessels following middle cerebral artery occlusion in baboons, the consequences of endothelial P-selectin expression in stroke have not been determined. To define the role of P-selectin in stroke, a murine model of focal cerebral ischemia and reperfusion consisting of intraluminal middle cerebral artery (MCA) occlusion for 45 minutes followed by 22 hours of reperfusion was used in two groups of mice; transgenic mice that were homozygous null for P-selectin (PS -/-), and wild type cousin controls (PS +/+). Cerebral infarct volumes were calculated from planimetered serial sections stained with triphenyltetrazolium chloride, and expressed as the percentage of infarcted tissue in the ipsilateral hemisphere. Neurologic outcome was based on animal behavior observed by a blinded investigator (1: no deficit; 2: circling; 3: spinning; 4: immobile). Ipsilateral cortical cerebral blood flow (CBF) was determined by laser doppler flowmetry and expressed as percent of contralateral cortical CBF. PS -/- mice showed a 3.8-fold reduction in infarct volumes compared with PS +/+ controls (7.6 ± 4.4% vs 29.2 ± 10.1%, $p<0.05$). This reduction in infarct volumes in mice devoid of P-selectin was mirrored by improved survival (87% vs. 42%, $p<0.05$) and a trend towards reduced neurological deficit (1.9 ±0.4 vs. 2.5 ±0.3, p=NS) in survivors. Because there was a tendency for increased cerebral blood flow following cerebral ischemia and reperfusion in the PS -/- cohort (65 ± 11% vs. 46 ± 18% for controls, p=NS), these studies suggest that P-selectin-dependent adhesion may contribute to cerebral no-reflow. Taken together, these data implicate an important role for P-selectin expression in the pathophysiology of stroke, and suggest novel pharmacologic strategies to improve stroke outcome.

EXAMPLE 6: Absence of the P-selectin Gene Reduces Post-ischemic Cerebral Neutrophil Accumulation, No-reflow, and Tissue Injury in a Murine Model of Reperfused Stroke

Recent studies in humans indicate that reestablishment of cerebral blood flow (CBF) during the early period following the onset of

stroke reduces neurologic sequelae. It was hypothesized that P-selectin (PS), an early-acting neutrophil (PMN) adhesion molecule expressed by hypoxic endothelium may have an important pathophysiological role in evolving, reperfused stroke. Preliminary studies were performed in a murine model of transient focal cerebral ischemia consisting of intraluminal middle cerebral artery occlusion for 45 minutes followed by 22 hours of reperfusion. In this model, mice which do not express the PS gene (PS -/-) have smaller infarct volumes, reduced neurological deficit scores, and improved survival compared to wild-type controls (PS +/+). The current studies were performed to further define PS-induced mechanism(s) of cerebral injury. PS +/+ mice (n = 6) given a ^{125}I-labeled anti-PS IgG prior to surgery demonstrated a 216% greater accumulation of the antibody in the ipsilateral hemisphere by 30 min of reperfusion compared with sham-operated animals (n = 6, $p < 0.001$) or with animals given nonimmune IgG and subjected to transient focal cerebral ischemia and 30 min of reperfusion (n = 4, $p < 0.03$). In PS +/+ mice, accumulation of PMNs begins early following the initiation of focal ischemia, and continues throughout the period of reperfusion (2-fold increase in ipsilateral/contralateral ^{111}In-PMN accumulation by 22 hours, n = 8, $p < 0.05$). PS -/- mice showed a 25% reduction in PMN accumulation into the ipsilateral hemisphere by 22 hours (n = 7, $p < 0.05$). The effect of PS expression on post-ischemic cerebral no-reflow was investigated by measuring ipsilateral CBF serially during stroke evolution. Although baseline, post-occlusion, and initial reperfusion CBFs were identical, CBFs at 30 minutes of reperfusion were significantly greater in PS -/- mice (n = 5) compared to PS +/+ mice (n = 8, 2.4-fold greater, $p < 0.05$). This difference was sustained during the remainder of the 22 h reperfusion period. These data support an important early role for PS in PMN recruitment, post-ischemic no-reflow, and tissue damage in evolving stroke. This is the first demonstration of a pathophysiological role for PS in cerebral reperfusion injury, which suggests that PS blockade may represent a therapeutic target for the treatment of

reperfused stroke.

EXAMPLE 7: Carbon Monoxide and Evolving Stroke.

Carbon monoxide gas, a toxic byproduct of heme catabolism, is involved in long-term potentiation and memory in the central nervous system. However, other physiologic roles for CO production in the brain are unknown. Because heme oxygenase is induced during inflammatory conditions, it was investigated whether endogenous CO production may confer a cerebral protective role in stroke. In a murine model of focal cerebral ischemia, heme oxygenase type I was induced at the mRNA (by Northern blot) and protein levels (by Western blot), localized to the cerebral vascular endothelium in the ischemic hemisphere by in situ hybridization and immunohistochemistry. Local production of CO by direct measurement was observed in the ischemic zone. In parallel experiments, murine brain endothelial cells exposed to a hypoxic environment demonstrated similar induction of heme oxygenase mRNA, protein and CO generation. To determine whether CO production was incidental to the pathophysiology of stroke, CO production was blocked by tin protoporphyrin administration (confirmed by direct measurement of reduced local CO levels). These animals demonstrated significantly larger infarct volumes, worse neurological outcomes, and increased mortality compared with untreated controls. Furthermore, administration of CO prior to stroke conferred significant cerebral protection. As this protection was not observed in animals treated with biliverdin, the coincident byproduct of heme catabolism, these data suggest that endogenous CO production per se has a protective role in evolving stroke.

Introduction

There is a considerable body of literature and a common recognition of the toxic effects of exogenous carbon monoxide (CO), which binds avidly to heme centers, inhibiting oxygen transport and poisoning cellular respiration. For many years, CO was regarded as an incidental byproduct of heme catabolism, but recent data in the

brain suggests that CO produced in discrete neurons by heme oxygenase II may modulate long-term potentiation. In rats, exposure to heat shock has been correlated with the expression of a 32 kDa heat shock protein (heme oxygenase I) in several organs including the brain. The physiological significance of this HSP32 induction has been teleologically ascribed to the stoichiometric liberation of CO by heme oxygenase I (HOI). In most, experimental studies, HOI induction serves only as an incidental marker of cellular oxidant stress. The recent identification of an anti-inflammatory role for HOI in a model of peritoneal inflammation has been ascribed to the production of the natural antioxidant biliverdin during the process of heme catabolism.

The current study reports for the first time that the postischemic brain generates enormous quantities of CO. Using a murine model of focal cerebral ischemia in which the middle cerebral artery is occluded by an intraluminal suture, HOI production in the ischemic hemisphere was increased significantly in comparison to the nonischemic hemisphere. Because immunohistochemistry and in situ hybridization localized the source of HOI to endothelial cells within the ischemic hemisphere, an in vitro model of cellular hypoxia was used to confirm the induction of HOI message, protein and activity in murine cerebral microvascular endothelial cells. Blockade of CO production using tin or zinc protoporphyrin IX was associated with an increase in cerebral infarct volume and mortality, whereas exposing animals to CO immediately prior to ischemia conferred significant dose-dependent cerebral protection within a narrow therapeutic window. Biliverdin administration was without effect in this model. Taken together, these data indicate that ischemic brain tissue produces large amounts of CO, the production of which confers cerebral protection that limits the amount of tissue destroyed during stroke.

<u>Methods</u>

Protoporphyrin preparation and administration. Tin protoporphyrin IX dichloride (20 mg, Porphyrin Products, Logan, UT), zinc

protoporphyrin IX (17 mg, Porphyrin Products, Logan, UT), or biliverdin (18 mg, Porphyrin Products, Logan, UT) was initially dissolved in dimethyl sulfoxide (2 mL). An aliquot of this solution (200 μL) was added to normal saline (9.8 mL) and this mixture was vortexed vigorously to yield a 2.7 X 10^{-4} M solution of the protoporphyrin. The solution container was wrapped in aluminum foil to prevent photolysis of the protoporphyrin and stored at 4 °C until used.

Micro-osmotic pumps (#1003D, Alza Corp., Palo Alto, CA) were loaded with this protoporphyrin solution (91 μL/pump) and implanted subcutaneously in the anesthetized mouse via a 1 cm dorsal midline incision 24 h prior to the start of surgery. These pumps administer drug solution at a rate of 0.95 $\pm$ 0.02 μL/h. At the time of surgery an additional dose of the protoporphyrin solution was administered (0.3 mL, i.v.) prior to insertion of the intralumenal occluding catheter. Each animal received the following total (injection + pump) drug amounts over the course of the study: tin protoporphyrin (0.070 mg), zinc protoporphyrin (0.059 mg), or biliverdin (0.061 mg).

EXAMPLE 8:

Hypoxia or free radicals induce P-selectin (PS) translocation to the endothelial cell (EC) surface, where it participates in neutrophil (PMN) adhesion during reperfusion. To explore a mechanism whereby nitrovasodilators may attenuate postischemic leukosequestration, we tested whether stimulating the NO/cGMP pathway could attenuate surface PS expression in hypoxic human umbillical vein ECs. ECs exposed to hypoxia (pO2< 20 Torr for 4 hours) demonstrated a 50% increase in νWF release ($p < 0.005$) (νWF is packaged with PS), paralleled by an 80% increase in surface PS expression ($p < 0.0001$), measured by specific binding of a radiolabeled anti-PS antibody. Under similar conditions, addition of the NO donor 3-morpholino sydnonimine (SIN-1, 0.1 mM) or the cGMP analog 8-Bromo-cGMP (cGMP, 10 nM) caused a reduction in νWF

release; Control vWF, 11 ± 0.4 mU/mL; SIN-1, 9.1 ± 0.3 mU/mL; cGMP, 9.7 ± 0.2 mU/mL; $p < 0.005$ for both SIN-1 or cGMP vs Control. Compared with controls, SIN-1 or cGMP also reduced surface PS expression (40% and 48% decreases respectively, $p<0.005$ for each) Using an immunofluorescent adherence assay, both SIN-1 and cGMP reduced HL60 binding to hypoxic HUVECs (53% and 86% decrease vs. controls, $p < 0.05$ for each). Measurement of fura-2 fluorescence demonstrated that hypoxia increased intracellular calcium concentration [Cai], and that increased [Cai] could be blocked by cGMP. Neither SIN-1 nor cGMP could further reduce PS expression when ECs were placed in a calcium-free medium. These data suggest that stimulation of the NO/cGMP pathway inhibits PS expression by inhibiting calcium-flux in ECs, and identify this inhibition as an important mechanism whereby nitrovasodilators may decrease PMN binding in post-ischemic tissues.

EXAMPLE 9: Factor IXai

Factor IX is a clotting factor which exists in humans and other mammals, and is an important part of the coagulation pathway. In the normal scheme of coagulation, Factor IX is activated by either Factor XIa or a tissue factor/VIIa complex to its active form, Factor IXa. Factor IXa then can activate Factor X, which triggers the final part of the coagulation cascade, leading to thrombosis. Because Factor X can be activated by one of two pathways, either the extrinsic (via VIIa/tissue factor) or the intrinsic pathways (via Factor IXa), we hypothesized that inhibiting Factor IXa might lead to impairment of some forms of hemostasis, but leave hemostasis in response to tissue injury intact. In other words, it might lead to blockade of some types of clotting, but might not lead to excessive or unwanted hemorrhage. Factor IXai is Factor IX which has been chemically modified so as to still resemble Factor IXa (ant therefore, can compete with native Factor IXa), but which lacks its activity. This can "overwhelm" or cause a competitive inhibition of the normal Factor IXa-dependent pathway of coagulation. Because Factor IXa binds to endothelium and platelets

and perhaps other sites, blocking the activity of Factor IXa may also be possible by administering agents which interfere with the binding of Factor IXa (or by interfering with the activation of Factor IX).

In stroke and other ischemic disorders, there may be clinical benefit derived by lysing an existing thrombus, but there is also the potentially devastating complication of hemorrhage. In the current experiments, the mouse model of cerebral ischemia and reperfusion (stroke) was used. Mice received an intravenous bolus of 300 μg/kg of Factor IXai just prior to surgery. Strokes were created by intraluminal occlusion of the right middle cerebral artery. When stroke outcomes were measured 24 hours later, animals that had received Factor IXai had smaller infarct volumes, improved cerebral perfusion, less neurological deficits, and reduced mortality compared with controls which underwent the same surgery but which did not receive Factor IXai. It was also noted that the Factor IXai animals were free of apparent intracerebral hemorrhage. By contrast, intracerebral hemorrhage was occasionally noted in the control animals not receiving Factor IXai.

Table II.

	Control		Experimental		
	mean	sd	mean	sd	stats
weight	26.91	3.21	25.25	2.49	0.14
dopp	0.96	0.24	1.04	0.35	0.52
occ. dop 1	0.18	0.07	0.16	0.08	0.60
occ dop 2	0.40	0.22	0.43	0.20	0.68
reper dop	0.55	0.42	0.53	0.30	0.89
sac dop	0.38	0.25	0.75	0.31	0.02
grade	2.22	0.67	1.67	0.49	
I/C Ratio	1.18	0.20	1.08		
inf vol	21.16	25.14	3.47	12.03	0.0452

count	11		16		

What is claimed is:

1. A method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of a selectin antagonist in a sufficient amount over a sufficient time period to prevent white blood cell accumulation so as to treat the ischemic disorder in the subject.

2. The method of claim 1, wherein the selectin antagonist comprises a peptide mimetic, a nucleic acid molecule, a ribozyme, a polypeptide, a small molecule, a carbohydrate molecule, a monosaccharide, an oligosaccharide or an antibody.

3. The method of claim 2, wherein the antibody is a selectin antibody.

4. The method of claim 3, wherein the antibody is a polyclonal antibody or a monoclonal antibody.

5. The method of claim 1, wherein the selectin antagonist comprises nitroglycerin or an agent which stimulates the nitric oxide, adenosine 3',5'-cyclic monophosphate, cyclic AMP or cyclic GMP pathway.

6. The method of claim 1, wherein the pharmaceutically acceptable form comprises a selectin antagonist and a pharmaceutically acceptable carrier.

7. The method of claim 6, wherein the carrier comprises an aerosol, intravenous, oral or topical carrier.

8. The method of claim 1, wherein the white blood cell is a neutrophil, a monocyte or a platlet.

9. The method of claim 1, wherein the subject is a mammal.

10. The method of claim 9, wherein the mammal is a human.

11. The method of claim 1, wherein the ischemic disorder comprises a peripheral vascular disorder, a pulmonary embolus, a venous thrombosis, a myocardial infarction, a transient ischemic attack, unstable angina, a reversible ischemic neurological deficit, sickle cell anemia or a stroke disorder.

12. The method of claim 1, wherein the subject is undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery.

13. The method of claim 12, wherein the organ transplantation surgery comprises heart, lung, pancreas or liver transplantation surgery.

14. The method of claim 1, wherein the selectin is a P-selectin, an E-selectin or an L-selectin.

15. A method for treating an ischemic disorder in a subject which comprises administering to the subject carbon monoxide gas in a sufficient amount over a sufficient period of time thereby treating the ischemic disorder in the subject.

16. The method of claim 15, wherein the administration is via inhalation or extracorporeal exposure.

17. The method of claim 15, wherein the amount comprises from about 0.0001% carbon monoxide in air to about 2% carbon monoxide in an inert gas.

18. The method of claim 17, wherein the inert gas comprises air, oxygen, argon or nitrogen.

19. The method of claim 15, wherein the amount comprises 0.1% carbon monoxide in air.

20. The method of claim 15, wherein the period of time comprises from about 1 day before surgery to about 1 day after surgery.

21. The method of claim 15, wherein the period of time comprises from about 12 hours before surgery to about 12 hours after surgery.

22. The method of claim 15, wherein the period of time comprises from about 12 hours before surgery to about 1 hour after surgery.

23. The method of claim 15, wherein the period of time comprises from about 1 hour before surgery to about 1 hour after surgery.

24. The method of claim 15, wherein the subject is a mammal.

25. The method of claim 24, wherein the mammal is a human.

26. The method of claim 15, wherein the ischemic disorder comprises a peripheral vascular disorder, a pulmonary embolus, a venous thrombosis, a myocardial infarction, a transient ischemic attack, unstable angina, a reversible ischemic neurological deficit, sickle cell anemia or a stroke disorder.

27. The method of claim 15, wherein the subject is undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery.

28. The method of claim 27, wherein the organ transplantation surgery comprises heart, lung, pancreas or liver

transplantation surgery.

29. A method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of inactivated Factor IX in a sufficient amount over a sufficient period of time to inhibit coagulation so as to treat the ischemic disorder in the subject.

30. The method of claim 29, wherein the amount comprises from about 75 μg/kg to about 550 μg/kg.

31. The method of claim 29, wherein the amount comprises 300 μg/kg.

32. The method of claim 29, wherein the pharmaceutically acceptable form comprises inactivated Factor IX and a pharmaceutically acceptable carrier.

33. The method of claim 32, wherein the carrier comprises an aerosol, intravenous, oral or topical carrier.

34. The method of claim 29, wherein the subject is a mammal.

35. The method of claim 34, wherein the mammal is a human.

36. The method of claim 29, wherein the ischemic disorder comprises a peripheral vascular disorder, a pulmonary embolus, a venous thrombosis, a myocardial infarction, a transient ischemic attack, unstable angina, a reversible ischemic neurological deficit, sickle cell anemia or a stroke disorder.

37. The method of claim 29, wherein the subject is undergoing heart surgery, lung surgery, spinal surgery, brain surgery, vascular surgery, abdominal surgery, or organ transplantation surgery.

38. The method of claim 37, wherein the organ transplantation surgery comprises heart, lung, pancreas or liver transplantation surgery.

39. A method for identifying a compound that is capable of improving an ischemic disorder in a subject which comprises:

a) administering the compound to an animal, which animal is a stroke animal model;

b) measuring stroke outcome in the animal, and

c) comparing the stroke outcome in step (b) with that of the stroke animal model in the absence of the compound so as to identify a compound capable of improving an ischemic disorder in a subject.

40. The method of claim 39, wherein the stroke animal model comprises a murine model of focal cerebral ischemia and reperfusion.

41. The method of claim 39, wherein the stroke outcome is measured by physical examination, magnetic resonance imaging, laser doppler flowmetry, triphenyl tetrazolium chloride staining, chemical assessment of neurological deficit, computed tomography scan, or cerebral cortical blood flow.

42. The method of claim 39, wherein the compound comprises a P-selectin antagonist.

43. A method for identifying a compound that is capable of preventing the accumulation of white blood cells in a subject which comprises:

a) administering the compound to an animal, which animal is

a stroke animal model;

b) measuring stroke outcome in the animal, and

c) comparing the stroke outcome in step (b) with that of the stroke animal model in the absence of the compound so as to identify a compound capable of preventing the accumulation of white blood cells in the subject.

44. The method of claim 43, wherein the white blood cell is a neutrophil, a monocyte or a platlet.

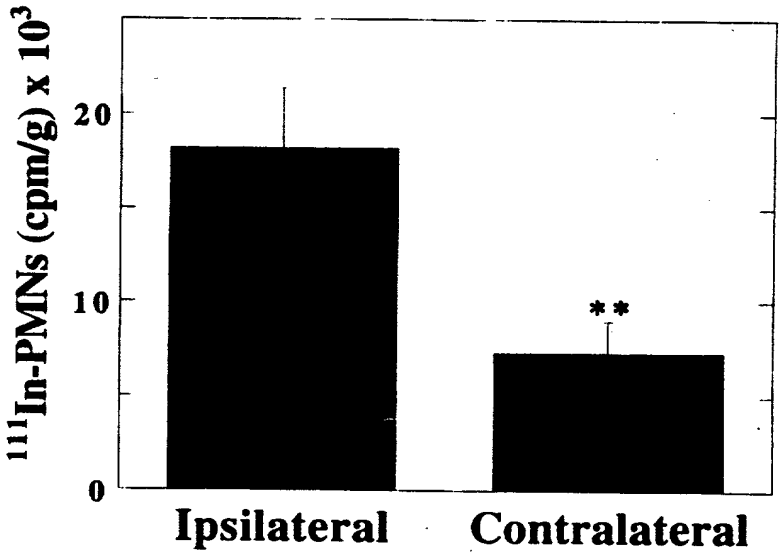
45. The method of claim 43, wherein the compound is a P-selectin inhibitor, a monocyte inhibitor, a platlet inhibitor or a neutrophil inhibitor.

METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

Abstract of the Disclosure

The present invention provides for a method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of a selectin antagonist in a sufficient amount over a sufficient time period to prevent white blood cell accumulation so as to treat the ischemic disorder in the subject. The invention further provides a method for treating an ischemic disorder in a subject which comprises administering to the subject carbon monoxide gas in a sufficient amount over a sufficient period of time thereby treating the ischemic disorder in the subject. The invention further provides a method for treating an ischemic disorder in a subject which comprises administering to the subject a pharmaceutically acceptable form of inactivated Factor IX in a sufficient amount over a sufficient period of time to inhibit coagulation so as to treat the ischemic disorder in the subject.

FIG. 1



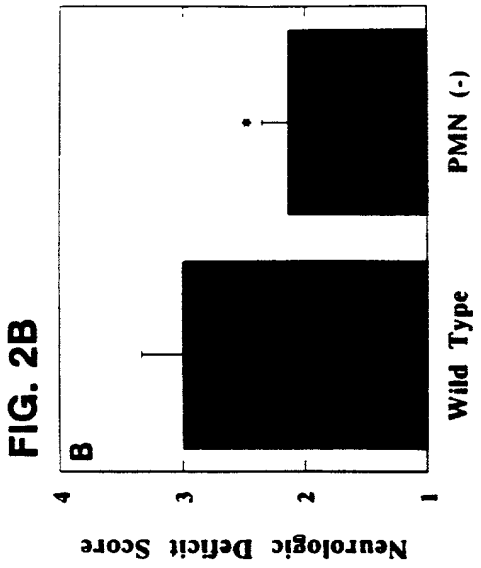


FIG. 2B
B
Neurologic Deficit Score
4
3
2
1
*
Wild Type
PMN (-)

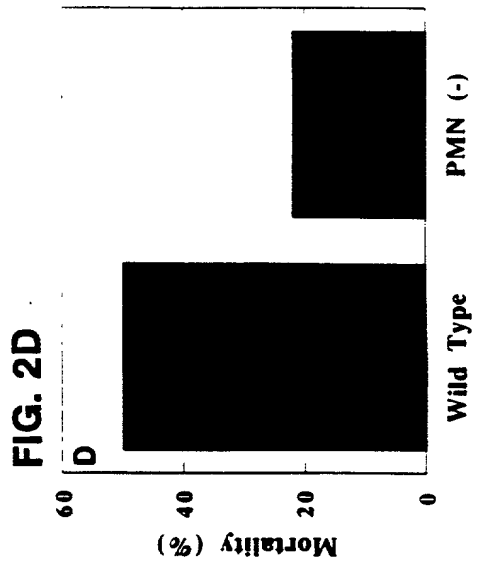


FIG. 2D
D
Mortality (%)
60
40
20
0
Wild Type
PMN (-)

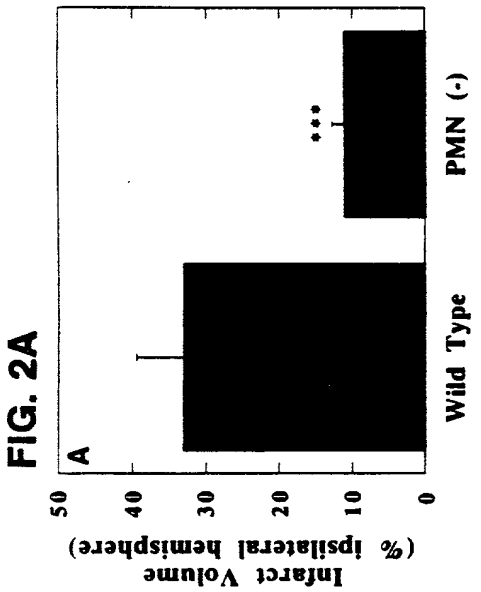


FIG. 2A
A
Infarct Volume
(% ipsilateral hemisphere)
50
40
30
20
10
0
***
Wild Type
PMN (-)

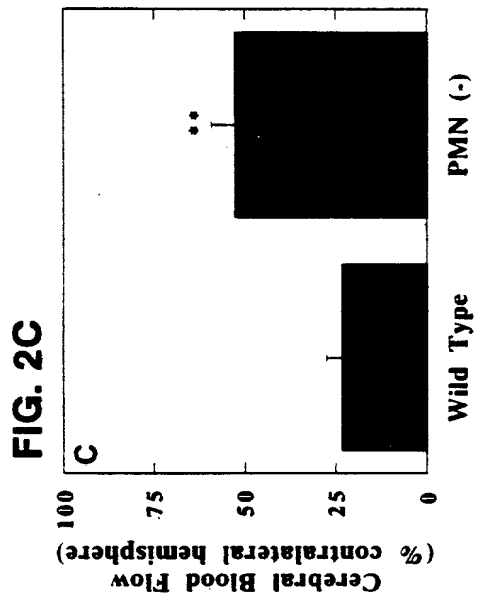


FIG. 2C
C
Cerebral Blood Flow
(% contralateral hemisphere)
100
75
50
25
0
**
Wild Type
PMN (-)

FIG. 3

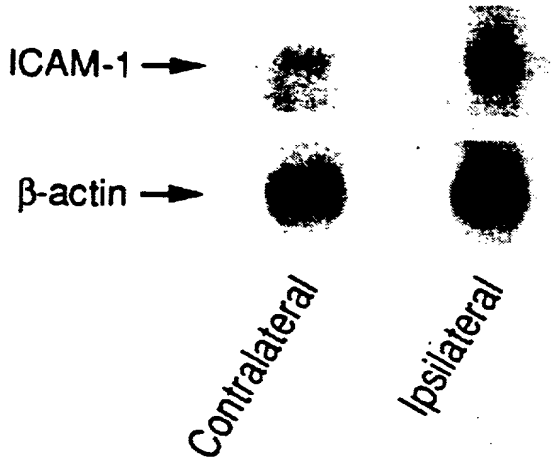


FIG. 4A

FIG. 4B

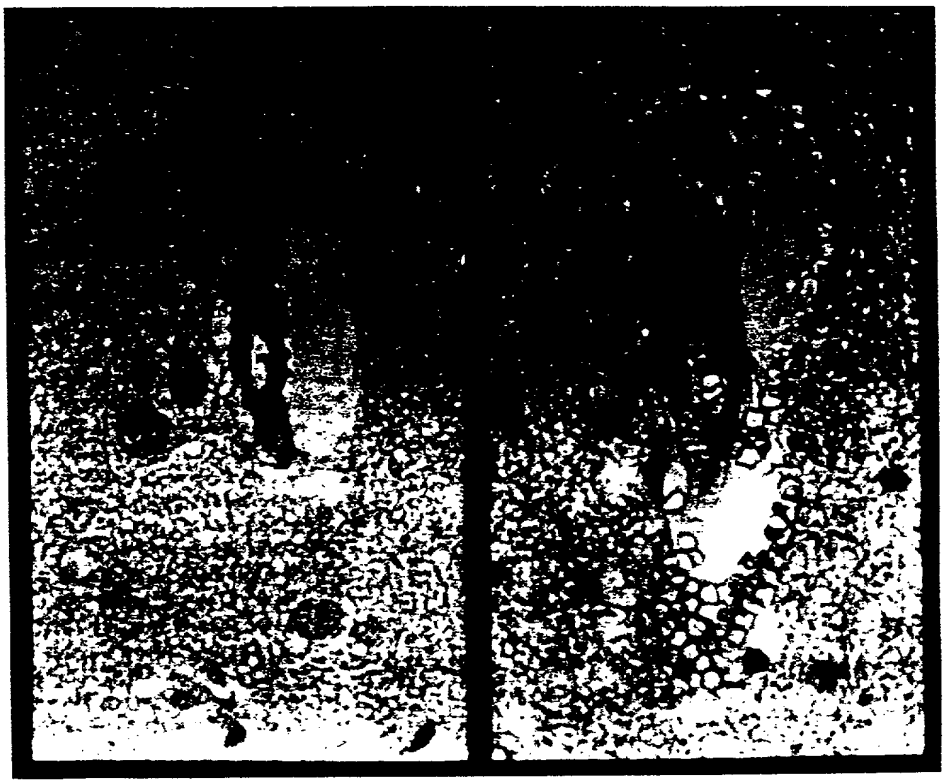
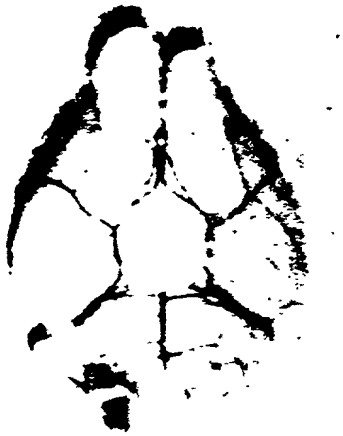
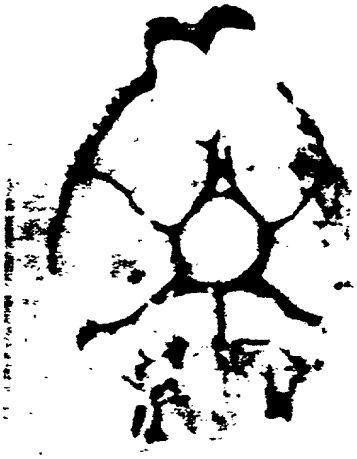


FIG. 5A



Wild Type

FIG. 5B



ICAM-1 (–/–)

FIG. 6A

Wild Type

FIG. 6B

ICAM-1 (–/–)

FIG. 7A

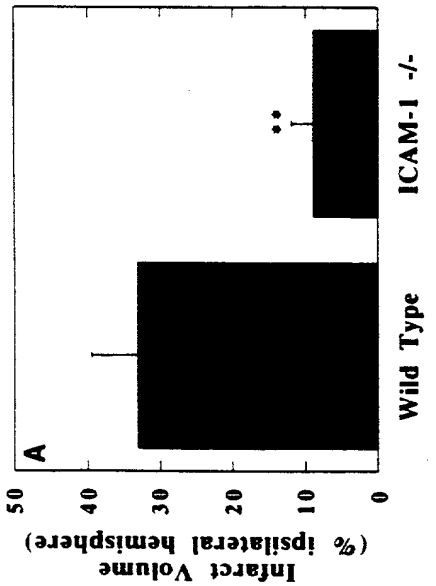


FIG. 7B

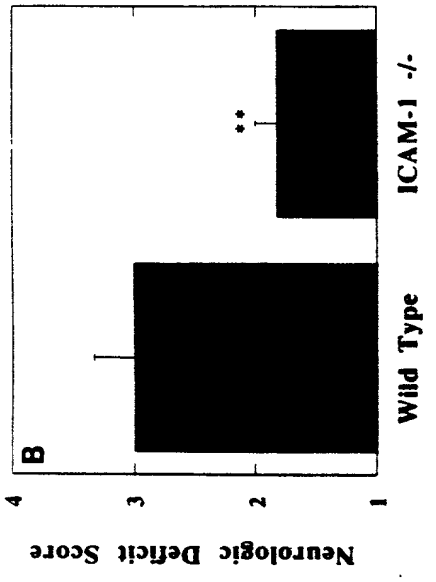


FIG. 7C

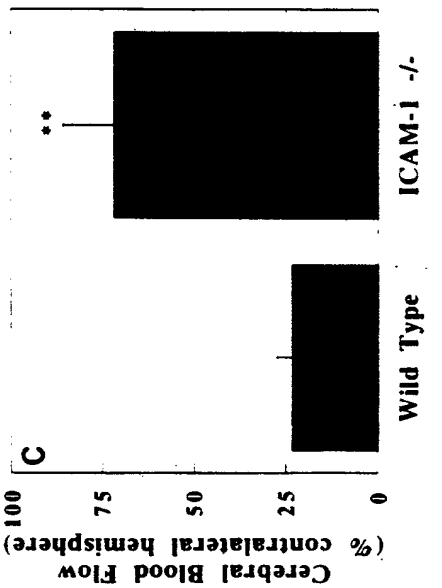


FIG. 7D

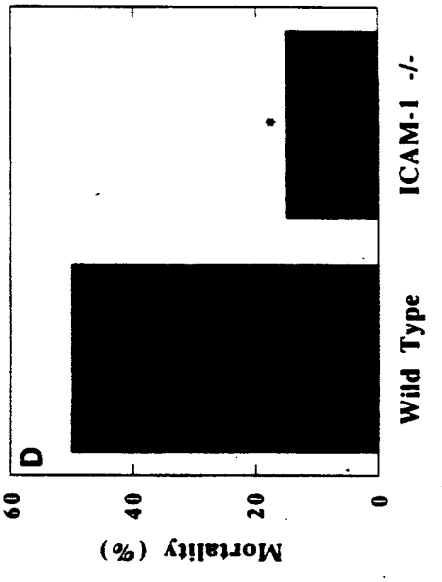


FIG. 8A

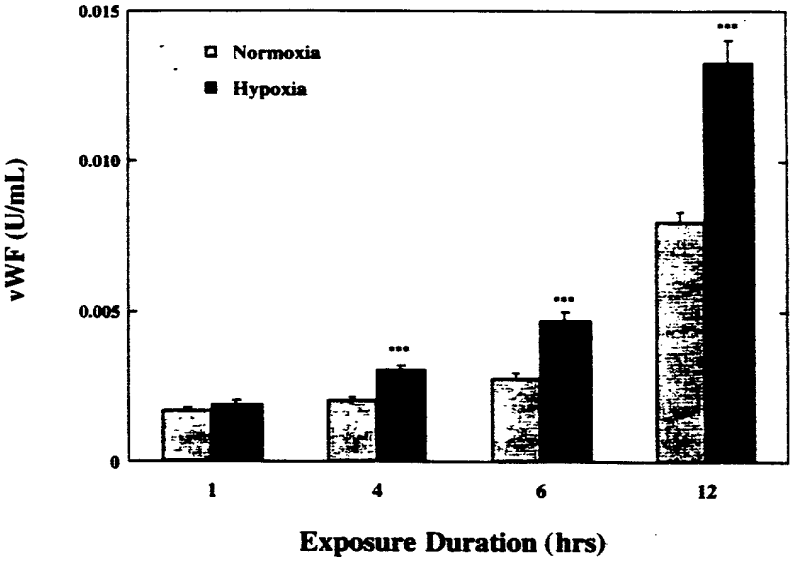

0.015
0.010
0.005
0
vWF (U/mL)
Normoxia
Hypoxia
***
***
***
1
4
6
12
Exposure Duration (hrs)

FIG. 8B

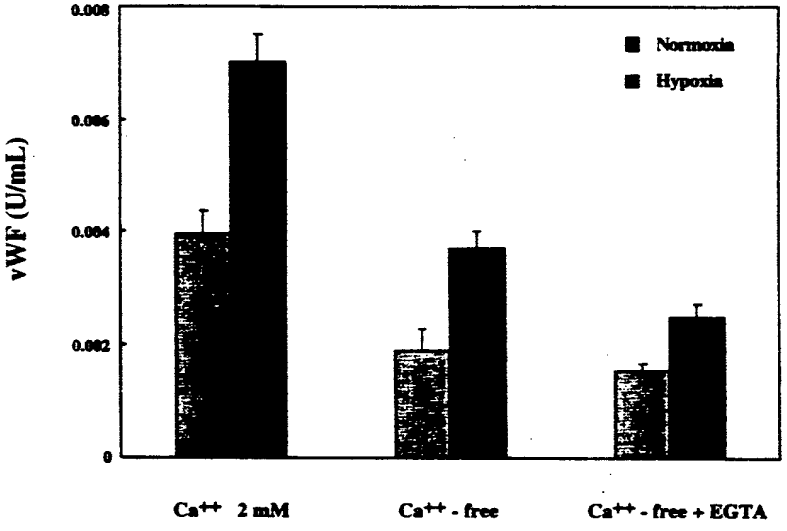

0.008
0.006
0.004
0.002
0
vWF (U/mL)
Normoxia
Hypoxia
Ca++ 2 mM
Ca++ - free
Ca++ - free + EGTA

FIG. 9A

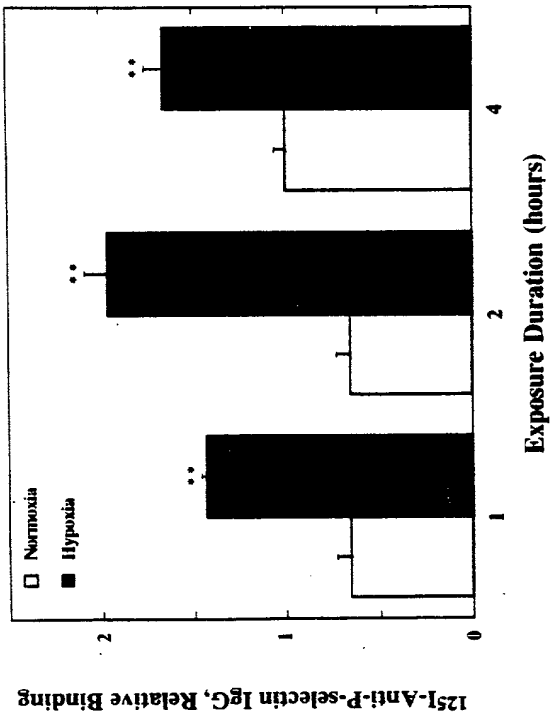


FIG. 9B

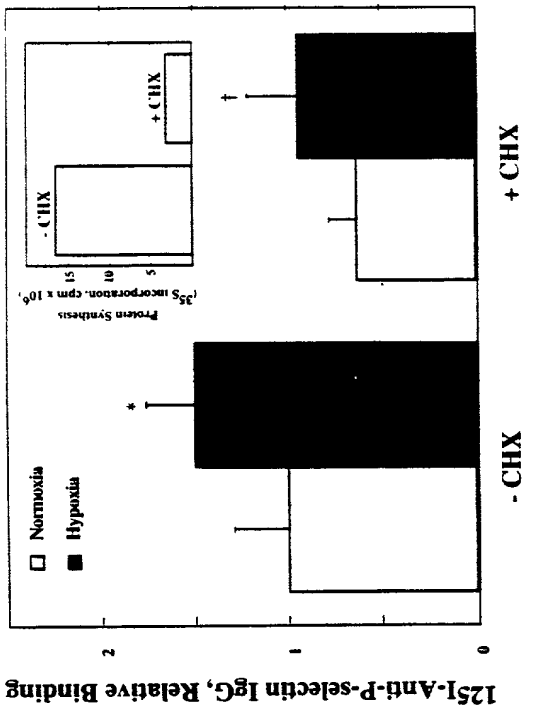


FIG. 9C

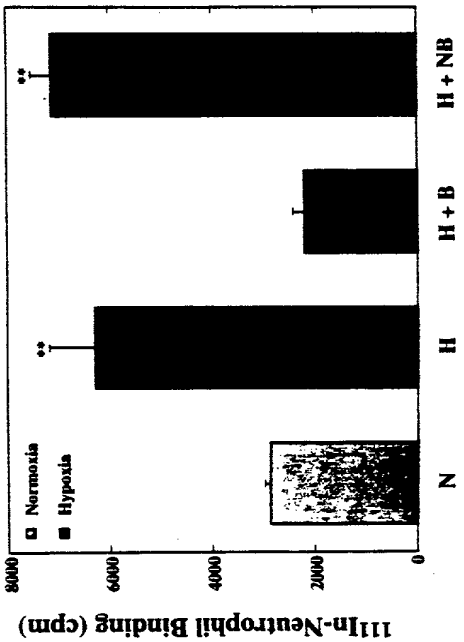


FIG. 10A

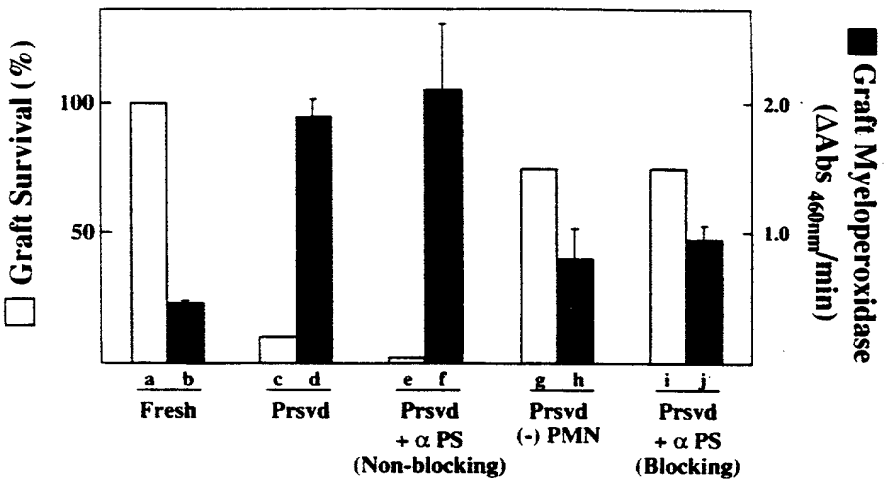


FIG. 10B

FIG. 10C

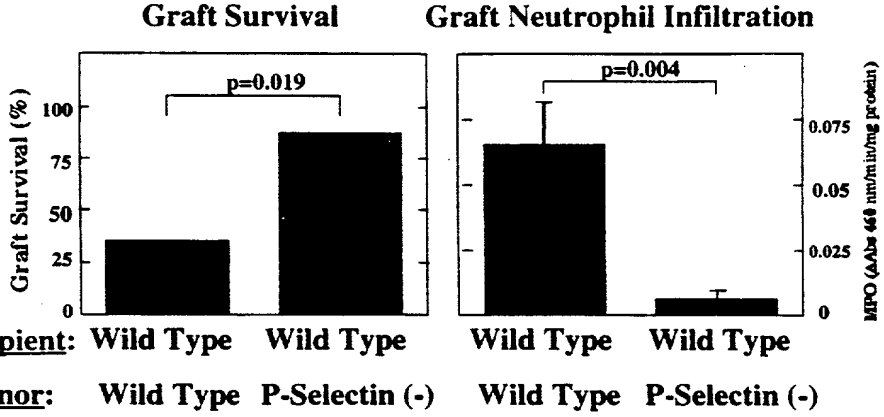


FIG. 11A

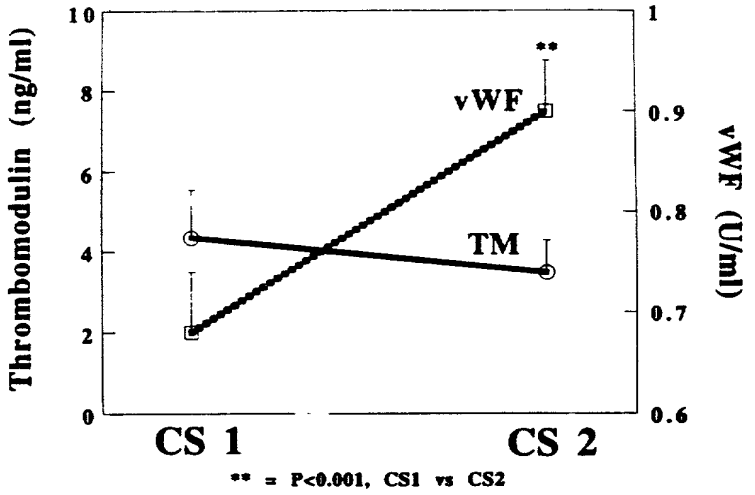

Thrombomodulin (ng/ml)
vWF (U/ml)
10
8
6
4
2
0
1
0.9
0.8
0.7
0.6
vWF
TM
**
CS 1
CS 2
** = P<0.001, CS1 vs CS2

FIG. 11B

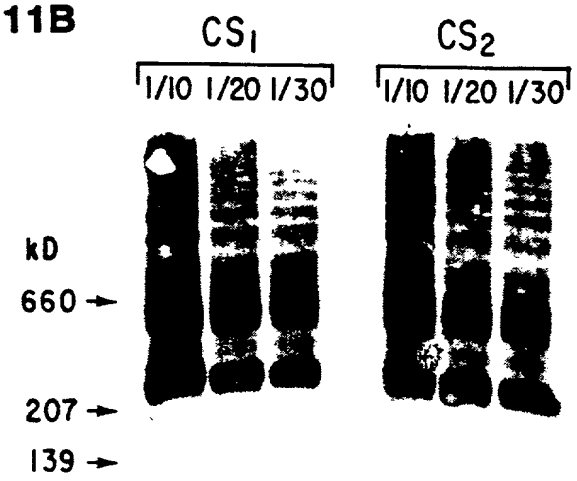


FIG. 12A

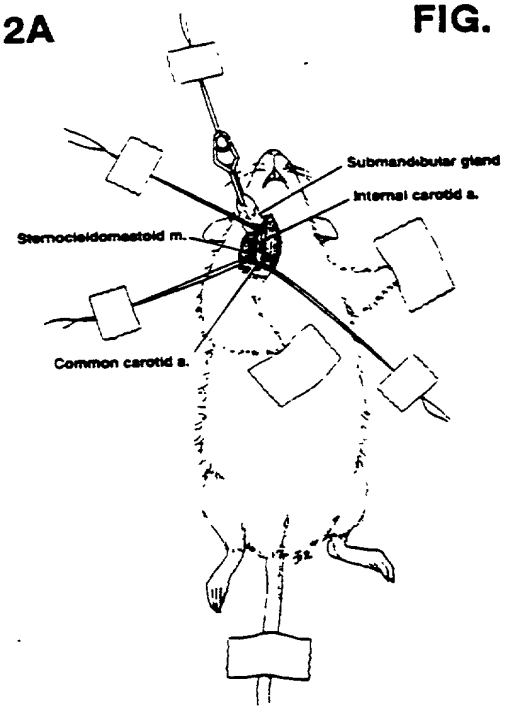


FIG. 12B

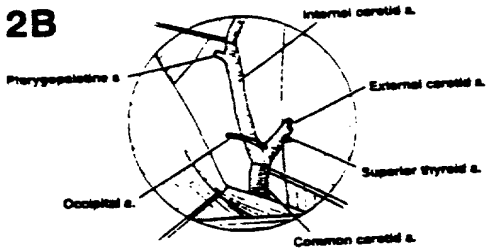


FIG. 12D

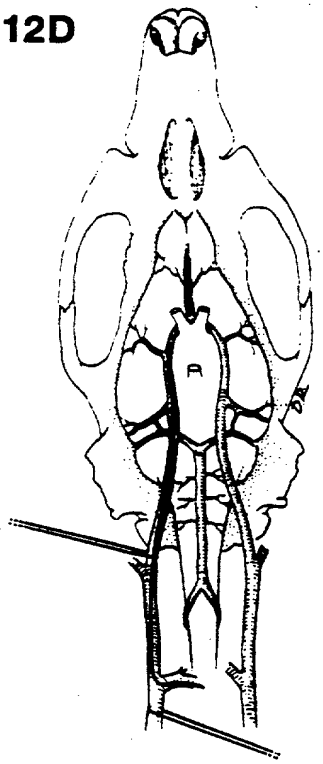


FIG. 12C

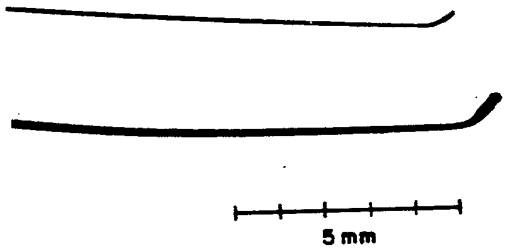


FIG. 13

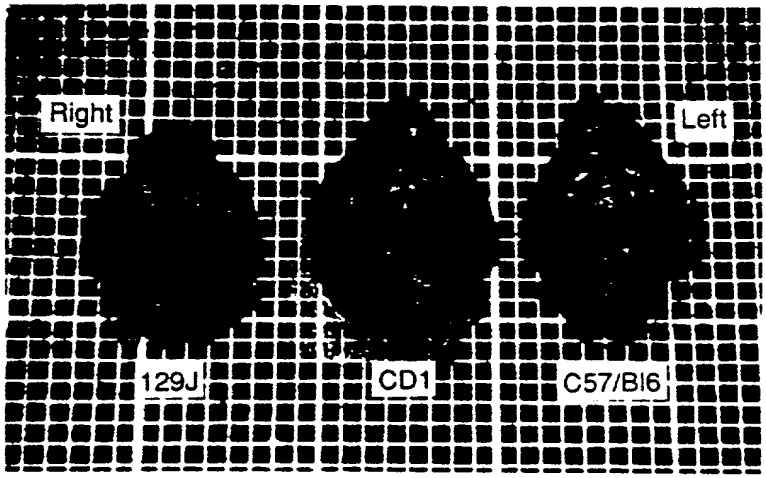


FIG. 14A

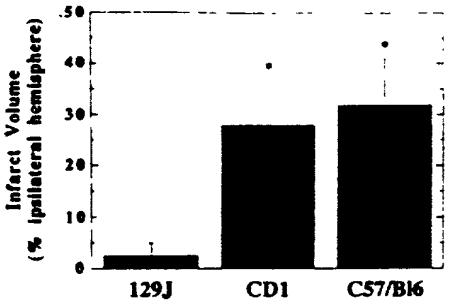


FIG. 14B

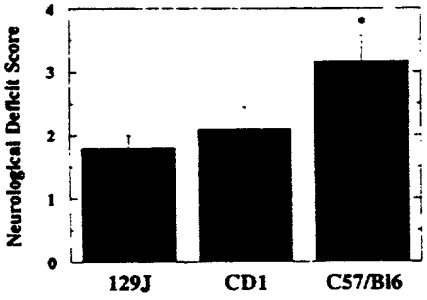


FIG. 14C

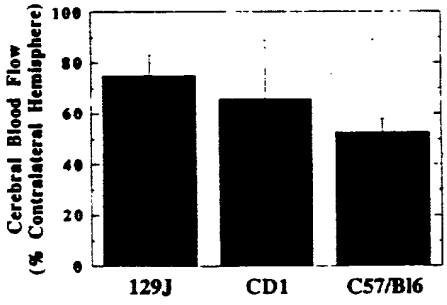


FIG. 15A

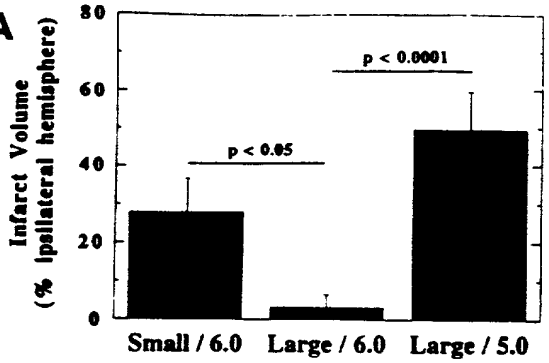


FIG. 15B

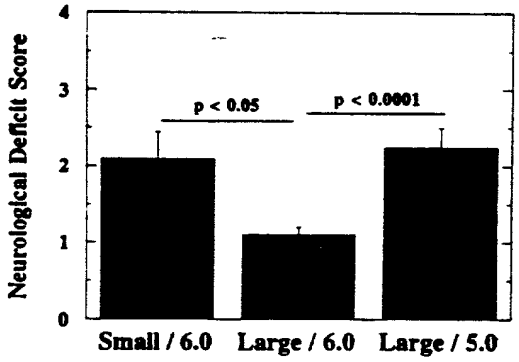


FIG. 15C

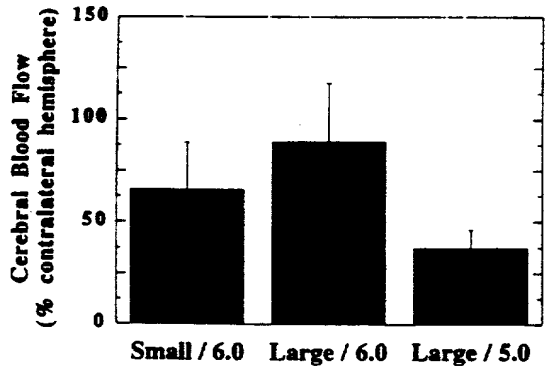


FIG. 16A

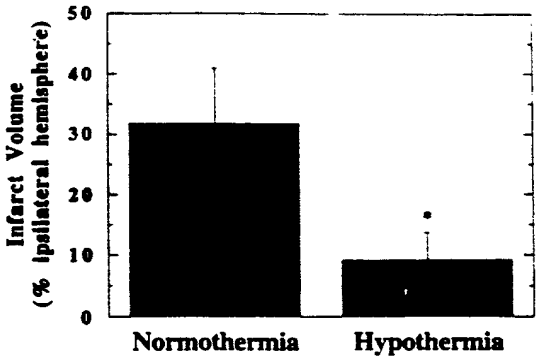


FIG. 16B

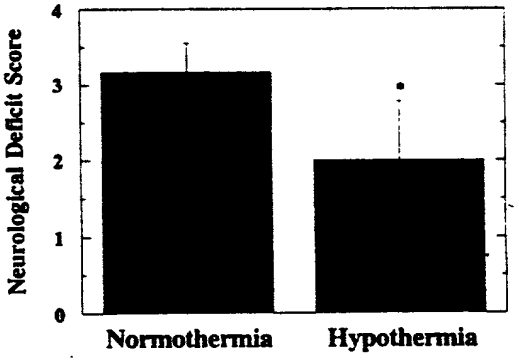


FIG. 16C

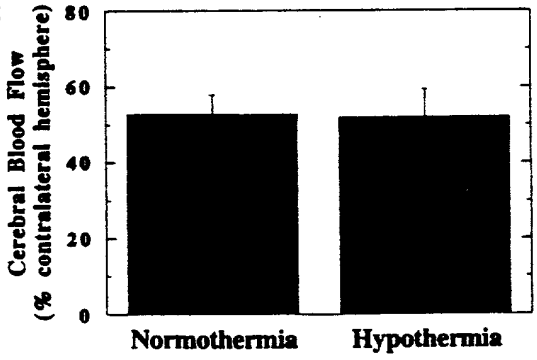


FIG. 17A

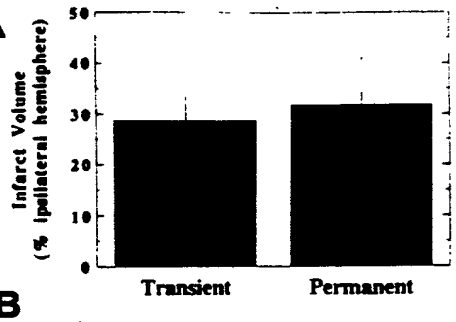


FIG. 17B

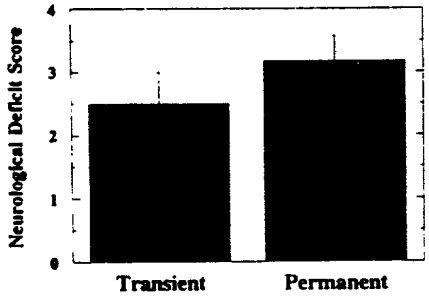


FIG. 17C

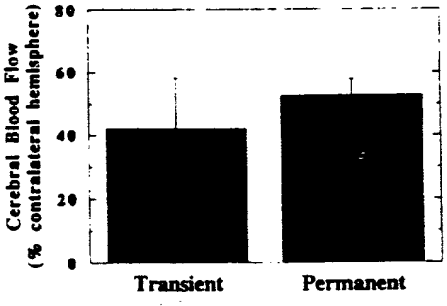


FIG. 18A

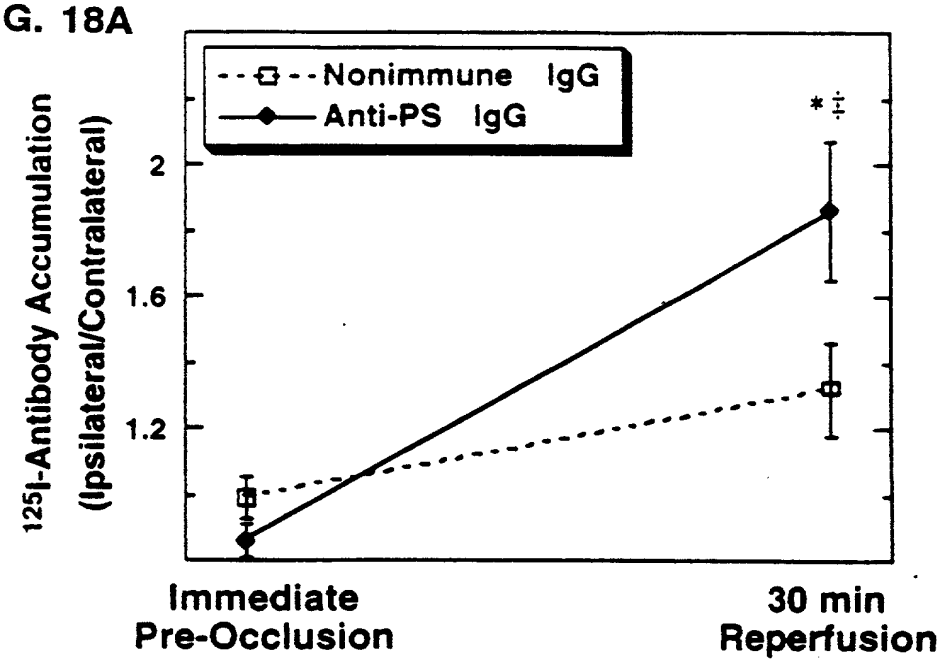


FIG. 18B

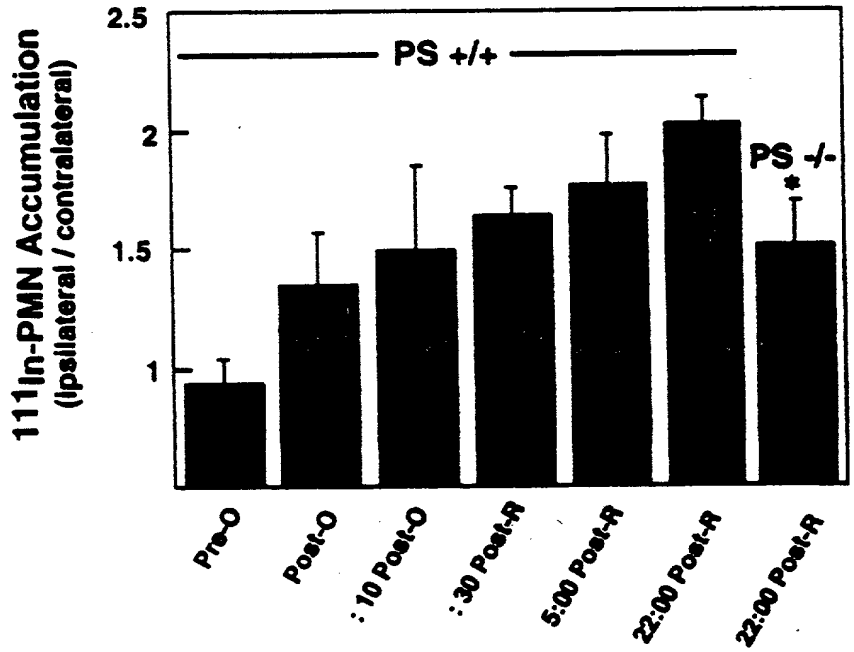


FIG. 19A

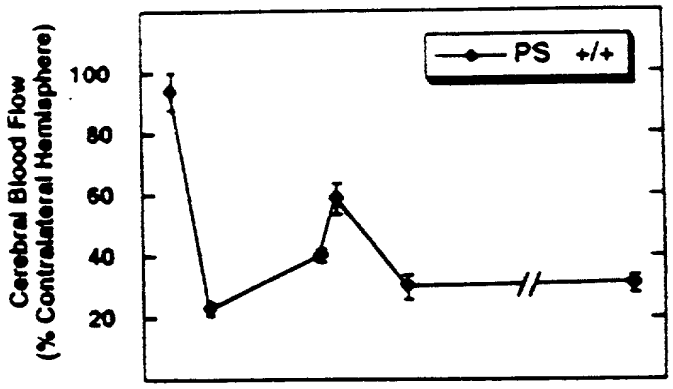


FIG. 19B

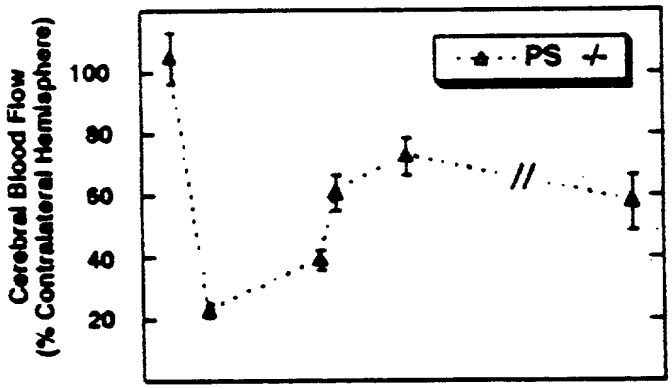


FIG. 19C

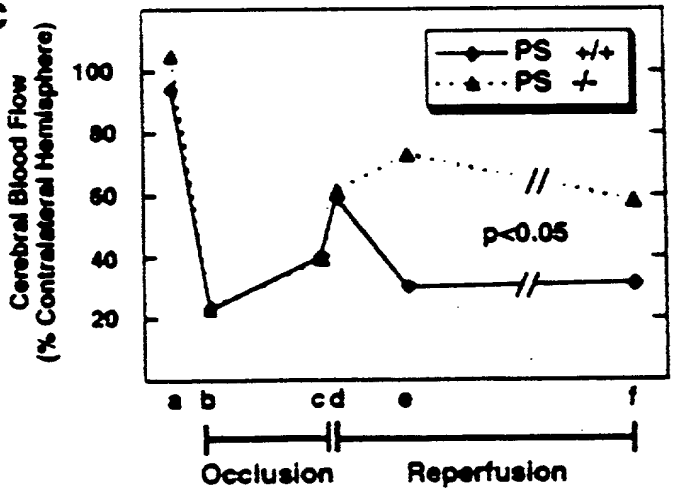


FIG. 20

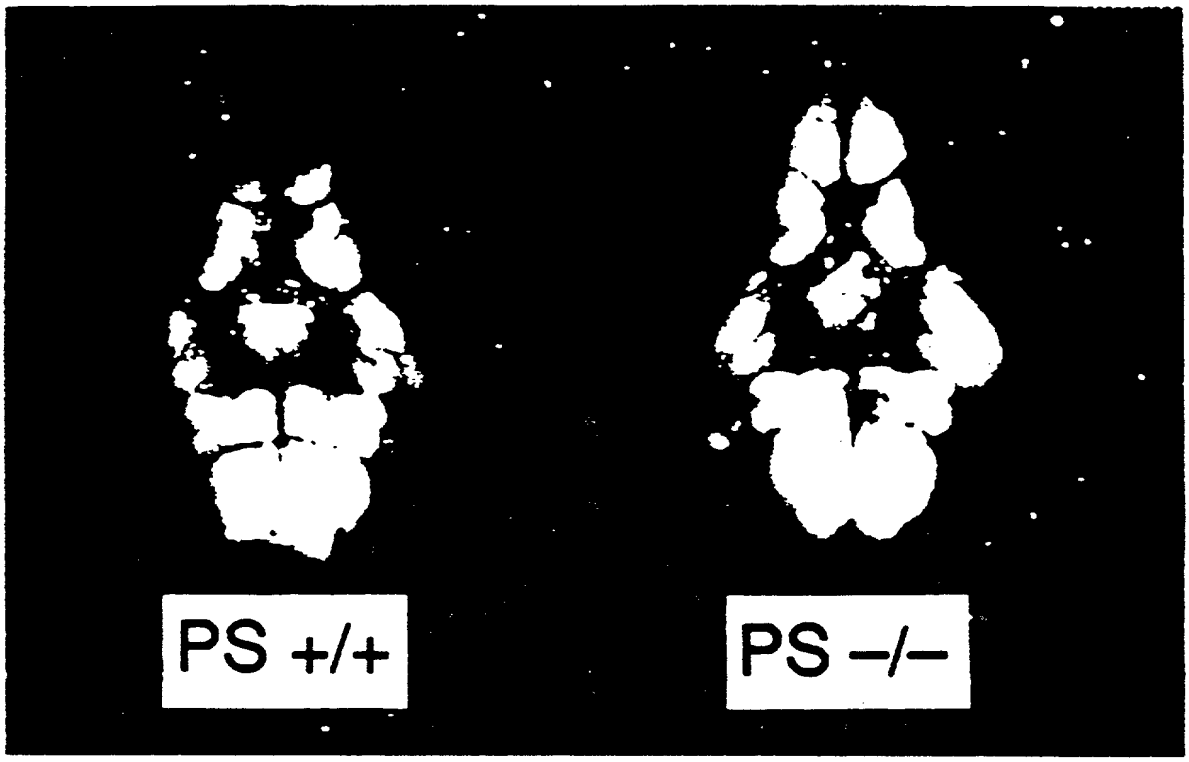


FIG. 21A

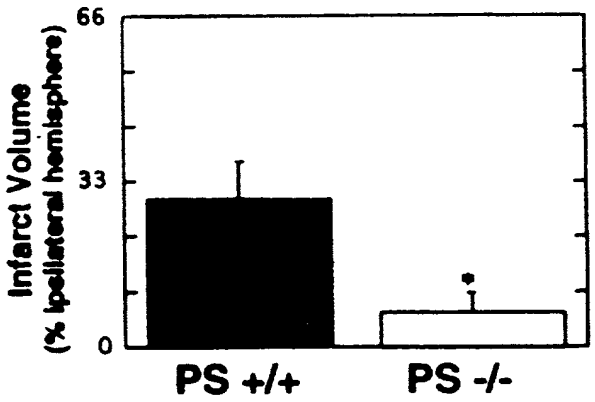


FIG. 21B

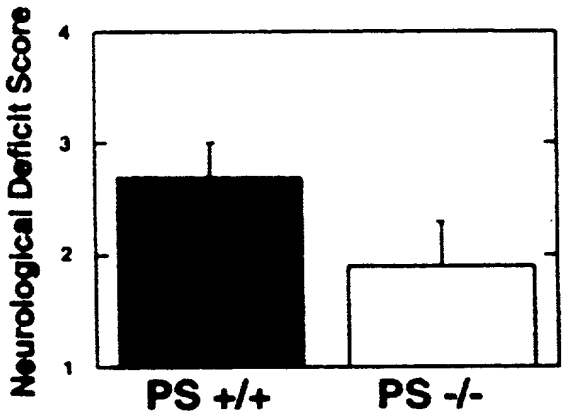
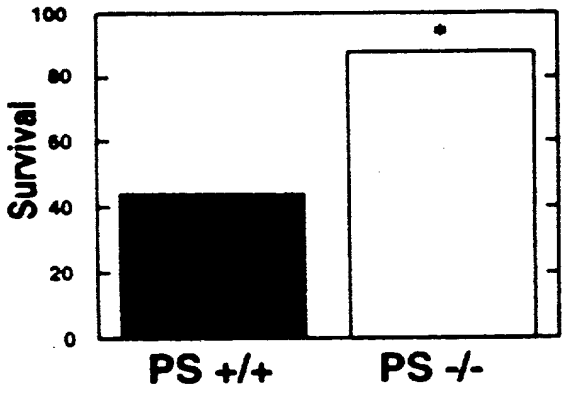


FIG. 21C



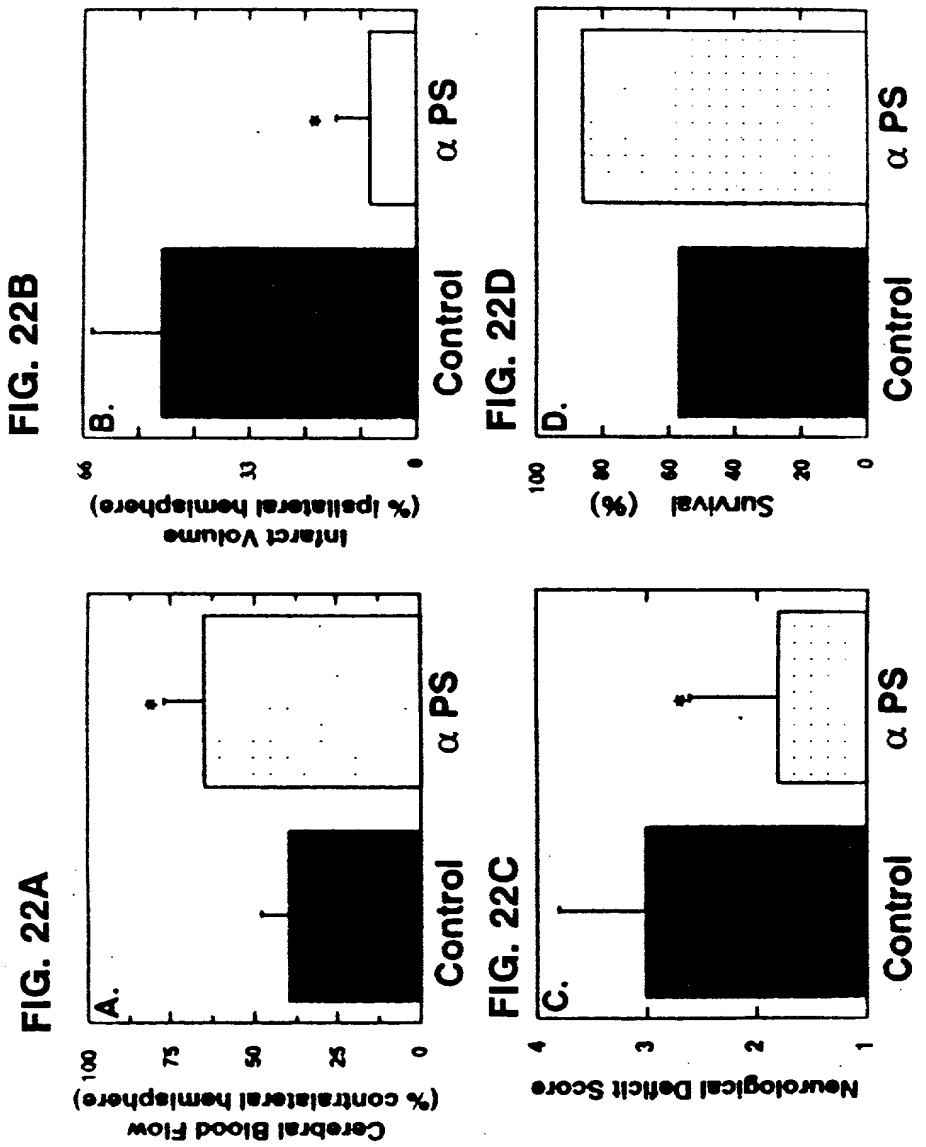


FIG. 23A

Effect of Inhaled CO on
Cerebral Infarct Volumes

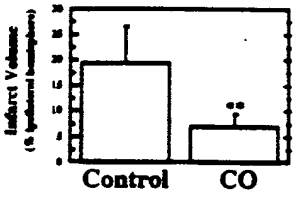


FIG. 23B

Effect of Inhaled CO on
Mortality Following Stroke

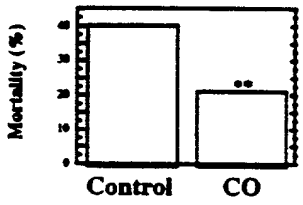
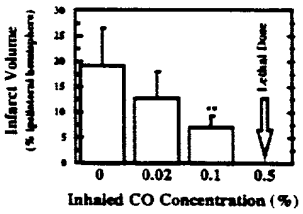


FIG. 24A

Effect of Inhaled CO on Cerebral Infarct Volumes: Dose-Response Relation



** p

FIG. 24B

Role of Heme Oxygenase In Stroke

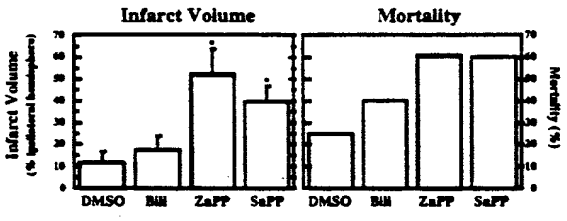
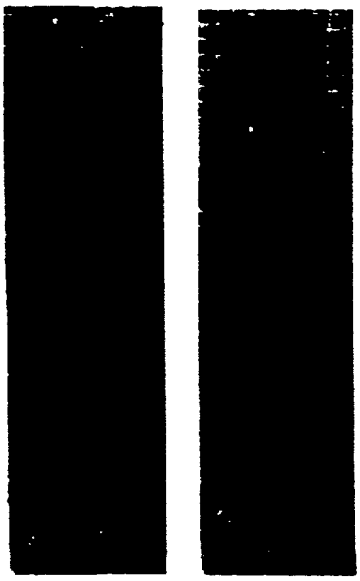


FIG. 25
Effect of Inhaled CO Pretreatment
on Cerebral Infarction Volume



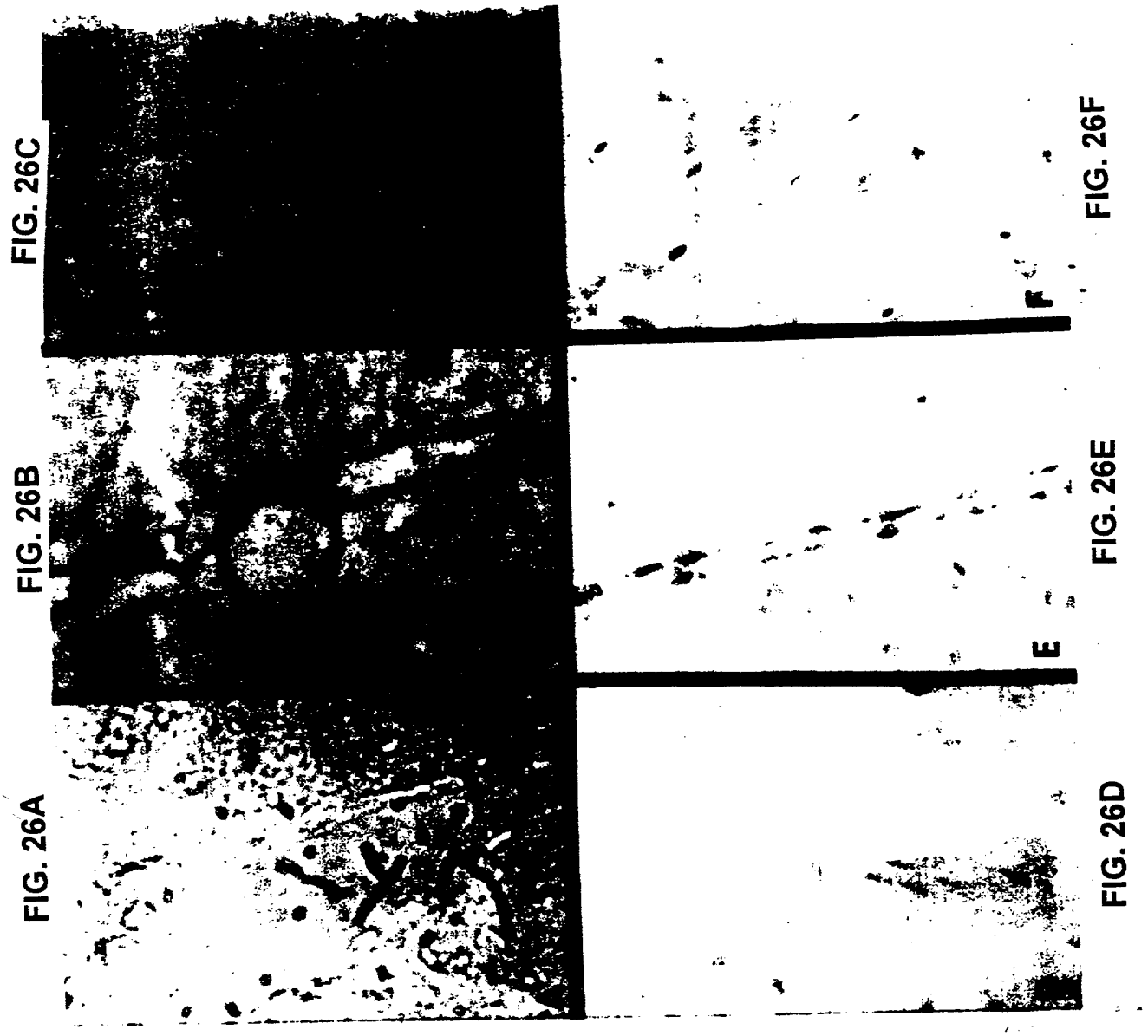


FIG. 27

Effect of Cerebral Ischemia
on Induction of HO-I

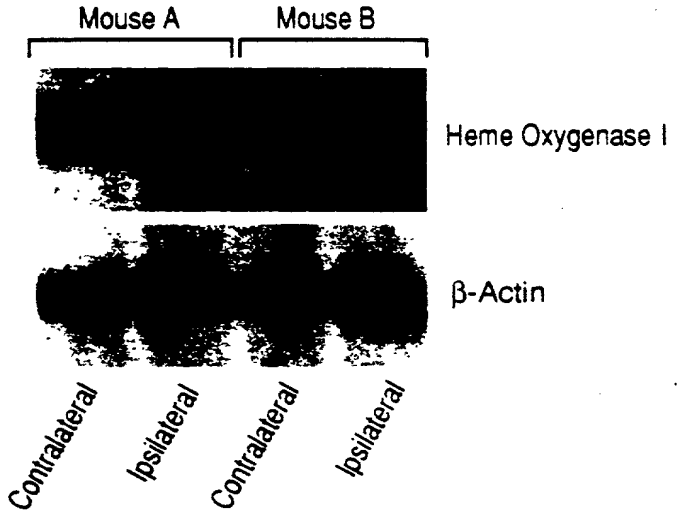


FIG. 28

Effect of Hypoxia on Cerebral HO Induction *In Vivo*

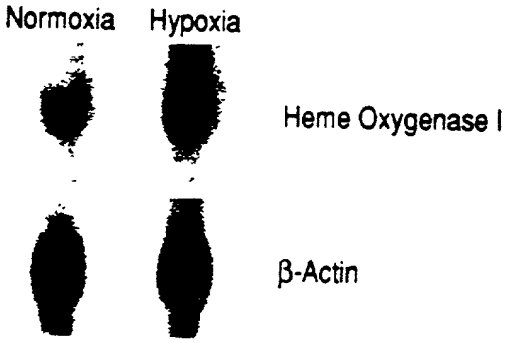


FIG. 29

Hypoxia-Induced Expression of HO-I Antigen
in Murine Brain Endothelial Cells

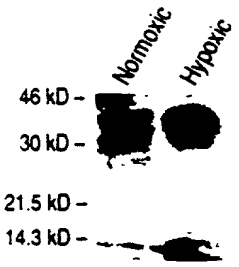
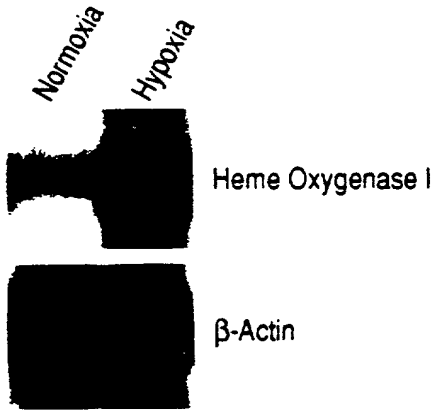


FIG. 30

Effect of Hypoxia on HO-I mRNA Induction
in Murine Brain Endothelial Cells



Applicant or Patentee: David J. Pinsky et al.
Serial or Patent No.: Not Yet Known
Filed or Issued: Herewith
Attorney's Docket No: 51917
Title of Invention or Patent: METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f) AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The Trustees of Columbia University in the City of New York

Address of Organization: 110 Low Memorial Library, West 116th & Broadway
New York, New York 10027

TYPE OF ORGANIZATION:

__X__ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
_____ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3)
_____ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
NAME OF STATE: _____
CITATION OF STATUTE: _____
_____ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
_____ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
NAME OF STATE: _____
CITATION OF STATUTE: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

by inventor(s) David J. Pinsky, David M. Stern, E. Sander Connolly Jr., Eric Rose, Ann M. Schmidt, Robert A. Solomon and Charles J. Prestigiacomo

described in:

__X__ the specification filed herewith
_____ application serial no. _____ filed _____
_____ patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below[a] and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

[a]NOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: N/A
Address: _____

_____ Individual _____ Small Business Concern _____ Nonprofit Organization

*See Reverse

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Jack M. Granowitz
Title In Organization: Executive Director, Columbia Innovation Enterprise
Address: Columbia University, Engineering Terrace -Suite 363, Amsterdam and 120th Street, New York, New York 10027
Signature: Jack M. Granowitz
Date Of Signature: Sept 25, 1996

*See Reverse

Docket No. 51917

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

45371 U.S. PTO
02/28/97

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS FOR TREATING AN ISCHEMIC DISORDER AND IMPROVING STROKE OUTCOME

the specification of which:
(check one)

___ *is attached hereto*

X *was filed on* September 27, 1996 *as*

Application Serial No 08/721,447

and was amended ______________________.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:

Prior Foreign Application(s)			*Priority Claimed*	
Number	*Country*	*Filing Date*	*Yes*	*No*
N/A				

Declaration and Power of Attorney *Page 2*

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

Provisional Application No.	*Filing Date*	*Status*
N/A		

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

Application Serial No.	*Filing Date*	*Status*
N/A		

And I hereby appoint

John P. White (Reg. No. 28,678); Thomas F. Moran (Reg. No. 16,579); Norman H. Zivin (Reg. No. 25,385); Ivan S. Kavrukov (Reg. No. 25,161); Christopher C. Dunham (Reg. No. 22,031); Thomas G. Carulli (Reg. No. 30,616); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Richard S. Milner (Reg. No. 33,970); Albert Wai-Kit Chan (Reg. No. 36,479); Kristina L. Konstas (Reg. No. 37,864); Mary Anne P. Tanner (Reg. No 40,197); and Mary Catherine DiNunzio (Reg.No. 37,306)

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and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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